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VIRULENCE TO MICE OF *STAPHYLOCOCCUS PYOGENES*: ITS MEASUREMENT AND ITS RELATION TO CERTAIN *IN VITRO* PROPERTIES.

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THE lack of a suitable test in animals has been a major difficulty in studies on the virulence of *Staph. pyogenes*. This drawback is probably due to the low virulence of this organism for animals, which makes it necessary to inoculate large numbers of staphylococci, usually by the intraperitoneal or intravenous route, if virulence is to be assessed by the killing power of the organism. The result of this procedure in rabbits and mice with various strains of staphylococci is that the animals either mostly die within 24 hours with a few surviving indefinitely, or all survive with little or no signs of infection. Although it may be said that this experimental infection has no resemblance to clinical staphylococcal infections, it has been of some value in the study of staphylococci as in the experiments of Christie, North and Parkin (1946), who have shown that there is a close relationship between haemolysin production and virulence to mice. The method, however, is crude, and does not lend itself to such measurement as would be necessary to assess the relative importance of the various products of *Staph. pyogenes* that may contribute to virulence.

The method to be described here is based on that used by McIntosh and Selbie (1941), who inoculated mice intramuscularly with the clostridia of gas gangrene to produce a local lesion and a rapidly fatal infection. The intramuscular injection of *Staph. pyogenes* also produces a local lesion but is rarely fatal and, as will be shown here, the size and progress of the lesions vary with different staphylococci and give an indication of relative virulence. The method is thus, in a way, similar to that used by Smith, Hale and Smith (1947), who injected staphylococci into the testicle of the guinea-pig and assessed virulence by the size of the lesions. Besides giving rise to lesions that are amenable to measurement, the method has the advantage over the previous methods used of causing an infection more closely resembling the natural infection in man.

MATERIAL.

The 75 strains of staphylococci selected for virulence tests were part of a collection of 182 strains of *Staph. pyogenes* that were isolated from routine specimens from the wards of the Middlesex Hospital. The selected strains were fully representative of the main collection except for the inclusion of all the 11 strains that had no detectable orange pigment, and a higher proportion of strains with low haemolytic titres to allow scope for factors other than haemolysin production to show their effects on virulence.

METHODS.

Haemolysin.

Following the method described by McFarlan (1938) cultures in tryptic digest broth incubated at 37° for 48 hr. in air containing about 30 per cent CO_2 were used for the titration of haemolysin. Two sets of serial 4-fold dilutions of each culture were prepared in saline. To each tube of one set was added one drop of a 10 per cent suspension of rabbit red cells in saline, and to each of the second one drop of a similar suspension of sheep red cells. The tubes were placed in a water-bath at 37° for 1 hr. and then at 4° for 18 hr. The results were then read, the titre being taken as the highest dilution showing at least 50 per cent haemolysis.

Coagulase tests with mouse plasma.

Reactions in the coagulase tube test described by Fisk (1940) were generally much weaker with mouse plasma than with human plasma and could usually be detected only after 6 or 24 hours' incubation. Only one strain gave a typical gel, whereas the other reactions showed only a fibrin veil sometimes extending throughout the medium but more usually lying on the bottom of the tube. The strength of the reaction was judged by the amount of fibrin clot and its tenacity when the tube was shaken after incubation for 24 hr.

In the slide coagulase test recommended by Williams and Harper (1946) the reactions with mouse plasma were similar to those obtained with human plasma. The strength of the reaction was judged by the speed of the reaction and the size of the clumps.

Fibrinolysin.

Fibrinolytic activity was estimated by a modification of the plate method described by Christie and Wilson (1941). Human plasma in 12 ml. amounts was heated at 60° for 40 min. to precipitate the fibrin, which was then centrifuged out and re-suspended in 10 ml. normal saline and vigorously shaken with glass beads. The 10 ml. of re-suspended fibrin were added to 90 ml. of digest broth agar at 55° and plates were poured. One plate was used for each strain, which was inoculated by a serial dilution method to give both confluent growth and isolated colonies. Fibrinolytic activity was designated as ++ when circular clearings surrounding isolated colonies were seen after incubation for 24 hr., and + when clearings round colonies did not appear till 48 hr. or when clearing of the medium was confined to the margin of confluent growth. Those strains showing no clearing at 48 hr. were regarded as negative.

Hyaluronidase.

Hyaluronidase production was estimated by a viscosity method based on that described by Harris, Abrams and Harris (1950). Tubes containing equal quantities of the supernatant fluid of 18-hr. cultures in tryptic digest broth and a 0·4 per cent solution of potassium hyaluronate (Allen and Hanburys, Ltd.) in normal saline buffered with phosphate to pH 7 were incubated in a water-bath at 37° for 30 min. and then heated for 10 min. at 60°. Control tubes containing no hyaluronate were treated similarly. The relative viscosities of the test and control mixtures were then measured by taking the average of 4 readings of the time of flow between graduations 0·6 and 0·2 in a 1 ml. graduated pipette held vertically in a retort stand.

The 75 strains were then divided into three groups, the first of 24 strains with differences in time of flow between the test and control mixtures of not more than 0·4 seconds, the second of 26 strains with differences of 0·5 to 1·1 seconds and the third of 25 strains with a difference greater than 1·1 seconds. These groups were designated as having hyaluronidase activity of +++, ++ and + respectively, indicating decreasing hyaluronidase activity.

Colour.

Streak cultures on milk agar plates were used for the determination of colour after incubation at 37° for three days and at room temperature for a further two days.

Gelatin liquefaction.

Stab cultures in 12·5 per cent gelatin in tryptic digest broth were incubated at room temperature for 5 days and the amount of liquefaction observed was expressed by plus signs.

Mannitol fermentation.

All strains produced acid within 48 hr. in a medium consisting of 1 per cent mannitol in peptone water with phenol red as indicator. Reactions were graded ++ and + according to whether acid was first observed at 24 hr. or 48 hr. respectively.

Penicillin sensitivity.

Penicillin sensitivity was measured by the 2-fold serial dilution method described by Selbie, Simon and McIntosh (1945), using an inoculum of about 1000 organisms. The results were expressed as the coefficient of resistance which is a multiple of the resistance of the standard Oxford staphylococcus.

Virulence tests.

Market albino mice from 20 to 30 g. in weight were used for the virulence tests. In each test 3 mice were inoculated intramuscularly in the posterior aspect of the left thigh with 0·2 ml. of a culture in tryptic digest broth after incubation in air for 18 hr. The mice were then examined at 1, 2, 4 and 7 days after inoculation, when measurement to the nearest mm. was made by calipers of the transverse width of the inoculated thigh of each mouse. By subtracting the width of the

untreated thigh, which was usually 5 mm., a figure was obtained indicating the size of the swelling caused by the staphylococcal infection. The measurements were discontinued at 7 days because of the onset of ulceration in mice with large lesions.

Whole cultures were used for inoculation in preference to washed suspensions because of the possibility that the staphylococci might be damaged by washing. This choice entailed the possibility that the inocula might contain the haemolysin against rabbit cells which, as will be shown here, is associated with a preponderating effect on the development of the infection. However, the cultures that were used for virulence tests, being incubated in air, contained little or no haemolysin, and the possible effects of preformed haemolysin were further excluded by performing virulence tests on Strains No. 13, 222, 251 and 252 with both the usual 18-hr. cultures in air and the 48-hr. cultures used for measuring haemolysin. Haemolysin was detected in none of the ordinary cultures, and was found in titres of 1/20 in two of the CO_2 cultures and 1/5 in the other two. The type of inoculum had no significant effect on the size or progress of the lesions produced by any of the four strains, and the average size of the lesions at 1, 2, 4 and 7 days respectively were 3·6, 5·3, 5·7 and 5·3 mm. in the 12 mice inoculated with ordinary cultures and 3·3, 5·6, 5·6 and 5·3 mm. in the 12 mice inoculated with cultures in CO_2.

RESULTS.

The relationships between haemolysin titres against rabbit cells and other *in vitro* properties are shown in Table I. It will be seen that higher haemolysin titres against rabbit cells are associated with higher haemolysin titres against sheep cells, greater production of fibrinolysin and hyaluronidase, more active liquefaction of gelatin, and weaker coagulase reactions in the slide test.

The results of the virulence tests are shown in Table II in relation to certain *in vitro* properties which will be shown to be correlated with virulence. It is evident that these tests have demonstrated great differences in the capacity of staphylococci to produce swelling of the thigh in mice. Only 4 mice died within a week after inoculation, each in a separate test, so that the results could be analysed solely on the size of the lesions.

Relationships between Virulence and In vitro *Properties.*

Haemolysin.

In Fig. 1 it will be seen that the average size of the swellings on the first day after inoculation increases with increasing rabbit cell haemolysin titre. There are also differences in the shape of the curves, the strains with higher haemolysin titres giving more progressive lesions whereas those with lower titres tend to show regression.

There is also a positive correlation between the haemolysin titre with sheep cells and virulence as is shown in Fig. 2. In the analysis of all 75 strains it will be seen that the swellings are larger and tend to be more progressive with strains having a sheep cell titre of at least 1/5 than with the other strains. It is to be noted however that only 20 of the 75 strains have a haemolytic titre with sheep cells of at least 1/5, and then only when the titre against rabbit cells is as high and often much higher (Table I). It is therefore necessary to allow for the

TABLE I.—*Relationship between Haemolysin Titre against Rabbit Red Blood Cells and Other* In vitro *Properties.*

	Number of strains with haemolysin titre against rabbit cells of—							
	1/1280.	1/320.	1/80.	1/20.	1/5.	1/1.	<1/1.	Total.
All strains	2	9	18	10	24	3	9	75
Sheep cell haemolysin titre:								
1/80	0	3	2	0	0	0	0	5
1/20	2	2	0	0	0	0	0	4
1/5	0	1	8	1	1	0	0	11
1/1	0	2	4	2	5	0	0	13
<1/1	0	1	4	7	18	3	9	42
Coagulase, mouse plasma :								
Tube, ++	1	2	6	3	8	1	1	22
+	1	7	12	7	16	2	8	53
Slide, +++	0	0	4	3	6	0	3	16
++	0	1	9	4	11	3	2	30
+	2	7	5	2	6	0	2	24
−	0	1	0	1	1	0	2	5
Fibrinolysin :								
++	2	5	9	4	6	0	3	29
+	0	1	4	3	15	2	4	29
−	0	3	5	3	3	1	2	17
Hyaluronidase :								
+++	2	4	8	4	2	0	4	24
++	0	3	4	4	10	1	4	26
+	0	2	6	2	12	2	1	25
Colour :								
Orange, ++	2	3	5	2	16	2	4	34
Orange, +	0	5	8	6	6	1	4	30
Yellow	0	0	4	1	1	0	1	7
White	0	1	1	1	1	0	0	4
Gelatin liquefaction :								
++	2	7	12	7	10	0	4	42
+	0	2	5	3	14	3	5	32
−	0	0	1	0	0	0	0	1
Mannitol fermentation :								
++	1	8	10	7	17	1	7	51
+	1	1	8	3	7	2	2	24
Penicillin coefficient :								
½ to 2	1	3	11	3	15	3	3	39
4 to 8	1	0	0	2	9	0	3	15
16 to 128	0	6	7	5	0	0	3	21

effect associated with the haemolysin titre against rabbit cells to show whether there is any independent correlation between the titre of haemolysin against sheep cells and virulence. This has been done first by excluding all strains with a rabbit cell titre of 1/20 or less, among which only two have sheep cell titres of more than 1/1, and secondly by restricting the analysis to strains with a rabbit cell titre of 1/80 (Fig. 2). In both these cases there is a correlation between the size of the lesions at one day and the sheep cell titre but no significant difference in the shape of the curves. It would therefore appear that the haemolysin titre against sheep cells is correlated with an effect additive to that associated with the

haemolysin titre against rabbit cells in determining the early development of the lesions.

TABLE II.—*Results of Virulence Tests in Mice in Relation to Certain* In vitro *Properties.*

Haemolysin titre. Rabbit cells.	Haemolysin titre. Sheep cells.	Slide coagulase mouse plasma.	Fibrinolysin.	Strain No.	Virulence. Average size of swelling on day— 1.	2.	4.	7.
1/1280	1/20	<+++	++	24	6	8	8	9
	"	"	"	168	5	6	7	8
1/320	>1/5	<+++	++	58	7	8	7	9
	"	"	"	144	6	7	7	7
	"	"	"	160	3	5	5	5
	"	"	<++	175	6	7	7	7
	"	"	"	221	10	13	12	10
	"	"	"	236	6	7	7	5
	<1/5	"	++	28	5	5	6	9
	"	"	"	80	7	10	9	9
	"	"	<++	170	7	7	10	7
1/80	>1/5	+++	<++	21	6	8	7	8
	"	"	"	151	6	7	6	6
	"	"	"	153	6	7	6	7
	"	<+++	++	25	3	4	4	4
	"	"	"	81	5	6	6	6
	"	"	"	95	3	3	4	4
	"	"	"	152	8	9	9	10
	"	"	"	248	7	8	7	7
	"	"	<++	96	3	6	6	6
	"	"	"	173	4	6	6	6
	<1/5	+++	++	20	7	8	8	8
	"	<+++	"	40	3	5	5	5
	"	"	"	44	2	3	3	2
	"	"	"	76	2	2	4	5
	"	"	<++	6	4	4	5	4
	"	"	"	33	4	5	5	5
	"	"	"	50	2	3	2	2
	"	"	"	55	1	2	2	1
1/20	1/5	<+++	<++	252	2	4	5	6
	<1/5	+++	"	9	3	4	3	2
	"	"	"	10	6	7	7	6
	"	"	"	34	3	5	3	3
	"	<+++	++	23	4	3	3	4
	"	"	"	43	3	5	4	3
	"	"	"	51	3	4	6	5
	"	"	"	68	2	3	3	4
	"	"	<++	47	2	4	3	3
	"	"	"	222	3	5	6	5
1/5	1/5	<+++	++	17	2	3	4	2
	<1/5	+++	<++	13	4	5	5	4
	"	"	"	26	5	6	4	4
	"	"	"	71	4	4	5	6
	"	"	"	99	4	5	5	5
	"	"	"	246	4	6	7	6
	"	"	"	251	5	7	7	6
	"	<+++	++	41	2	3	4	3
	"	"	"	66	2	3	2	2
	"	"	"	136	4	6	5	5
	"	"	"	174	3	5	6	5
	"	"	"	195	3	4	5	5

TABLE II—*contd.*

Haemolysin titre. Rabbit cells.	Haemolysin titre. Sheep cells.	Slide coagulase mouse plasma.	Fibrinolysin.	Strain. No.	Virulence. Average size of swelling on day— 1.	2.	4.	7.
1/5	„	„	<++	7	3	2	2	2
	„	„	„	31	3	4	3	3
	„	„	„	65	2	3	3	2
	„	„	„	75	4	4	3	3
	„	„	„	98	3	2	3	2
	„	„	„	100	2	2	2	2
	„	„	„	103	4	5	4	4
	„	„	„	133	2	2	2	2
	„	„	„	140	2	2	2	2
	„	„	„	179	4	4	4	3
	„	„	„	210	2	3	4	2
	„	„	„	238	2	3	4	3
1/1	<1/1	<+++	<++	5	0	0	0	0
	„	„	„	29	4	3	1	1
	„	„	„	106	2	3	1	1
<1/1	<1/1	+++	++	27	2	1	1	0
	„	„	<++	14	2	1	0	0
	„	„	„	36	1	0	0	0
	„	<+++	++	48	1	1	0	0
	„	„	„	211	2	1	0	0
	„	„	<++	18	1	0	0	0
	„	„	„	88	1	0	0	0
	„	„	„	150	1	1	0	0
	„	„	„	188	1	0	0	0

Comparison of Fig. 1 and the third part of Fig. 2 shows that the curve of the strains with a rabbit cell haemolysin titre of 1/80 and a sheep cell titre of less than 1/5 (Fig. 2) fits between the two adjacent curves of the strains with rabbit cell titres of 1/5 and 1/20 (Fig. 1). There would thus appear to be little difference in virulence between strains of rabbit cell titres 1/5, 1/20 and 1/80 if the 12 strains with a sheep cell titre of more than 1/5 are excluded. This procedure yields an intermediate group of 40 strains in which it can be presumed that differences in virulence are mainly associated with factors other than the production of haemolysin as detected by the methods used here.

Coagulase and fibrinolysin.

Although we have found that strains giving stronger reactions in the tube test with mouse plasma are somewhat more virulent, the difference is not significant. With regard to both the slide coagulase reaction with mouse plasma and fibrinolytic activity, significant differences in virulence have been detected only between strains giving the strongest *in vitro* reaction and the remaining strains. Since only two strains show strongly positive reactions in both *in vitro* tests, it has been possible to make a simultaneous analysis of the relationships of coagulase production and fibrinolytic activity to virulence. In Fig. 3 three groups of strains are analysed, the first consisting of all 75 strains, the second excluding the 11 strains with a rabbit cell titre of 1/320 or more which have a preponderance of strains of low coagulase and high fibrinolysin production (Table I), and the

third consisting of the intermediate group of 40 strains with haemolysin titres of 1/5 to 1/80 against rabbit cells and less than 1/5 against sheep cells.

It will be seen in all three parts of Fig. 3 that a strong coagulase reaction is associated with lesions that are larger on the first day, more progressive to the second day and more regressive after the second day. The curve of strains with high fibrinolytic activity, on the other hand, changes its position downwards from Parts I to III of Fig. 3 as allowance is made for the effect of haemolysin, and in Part III it is evident that there is no correlation between fibrinolysin and

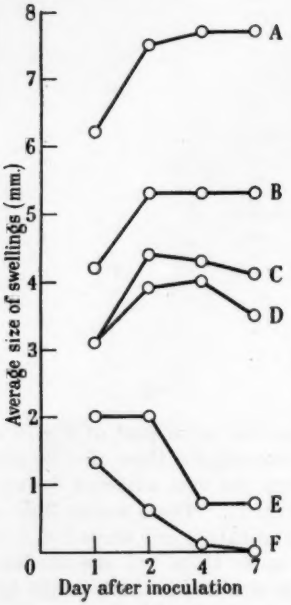

FIG. 1.—Relationship between haemolysin titre against rabbit cells and virulence to mice in 75 strains of *Staph. pyogenes*.

	Curve.					
	A.	B.	C.	D.	E.	F.
Haemolysin titre :	>1/320	1/80	1/20	1/5	1/1	<1/1
Number of strains :	11	18	10	24	3	9

the size of the lesion at one day. The shape of this curve, however, is the same in all three parts of Fig. 3, and shows that high fibrinolytic activity is associated with lesions that continue to be more progressive from the first day after inoculation.

Haemolysin in intermediate group of strains.

When allowance is made for the effects associated with coagulase and fibrinolysin a further analysis can be made of the effect associated with haemolysin

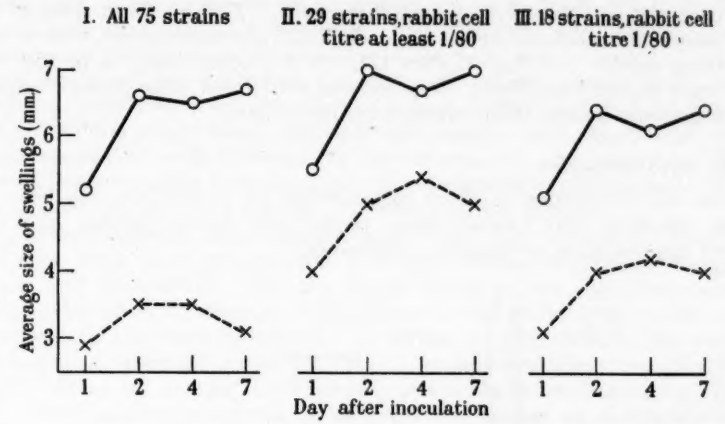
I. All 75 strains
II. 29 strains, rabbit cell titre at least 1/80
III. 18 strains, rabbit cell titre 1/80
Average size of swellings (mm.)
Day after inoculation

FIG. 2.—Relationship between haemolysin titre against sheep cells and virulence to mice of *Staph. pyogenes*.

	Sheep cell titre.	Number of strains in— Part I.	Part II.	Part III.
⊙——⊙	At least 1/5	20	18	10
×-----×	Less than 1/5	55	11	8

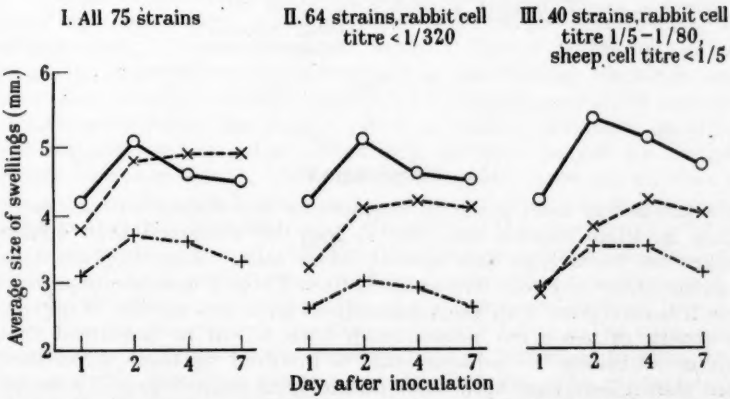
I. All 75 strains
II. 64 strains, rabbit cell titre < 1/320
III. 40 strains, rabbit cell titre 1/5 – 1/80, sheep cell titre < 1/5
Average size of swellings (mm.)
Day after inoculation

FIG. 3.—Relationships of slide coagulase reaction with mouse plasma and fibrinolytic activity to virulence of *Staph. pyogenes* to mice.

	Coagulase.	Fibrinolysin.	Number of strains in— Part I.	Part II.	Part III.
⊙——⊙	+++	< ++	14	14	9
×-----×	< +++	++	27	20	12
+—·—+	Remaining strains		34	30	19

in the intermediate group of 40 strains with haemolysin titres of 1/5 to 1/80 against rabbit cells and less than 1/5 against sheep cells. This has been done in Fig. 4, which shows the relationship between virulence and haemolysin titre in the 19 " remaining strains " of Fig. 3, Part III. It is evident here, as in Fig. 1, that strains with higher haemolysin titres against rabbit red cells produce larger and more progressive lesions than strains with lower titres.

Other in vitro *properties.*

Under the conditions of these experiments no correlation has been found between virulence and hyaluronidase production, colour, gelatin liquefaction, mannitol fermentation or penicillin resistance.

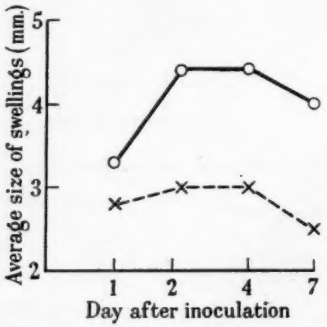

FIG. 4.—Relationship between haemolysin titre against rabbit cells and virulence to mice in " remaining strains " of *Staph. pyogenes* in Fig. 3, Part III.

	Number of strains of Rabbit cell titre.		
	1/80.	1/20.	1/5.
⊙——⊙	5	2	0
×-----×	0	0	12

DISCUSSION.

No attempt has been made to characterize the staphylococcal haemolysins concerned in these experiments, but it may be presumed that α-haemolysin determines the haemolytic titre against rabbit cells. Regarding the haemolytic titre against sheep red cells it is evident from Table I that the capacity to lyse sheep cells is correlated with the α-haemolysin titre, but in view of its variability within strains of the same α-haemolysin titre it can be presumed that some haemolysin additional to α-haemolysin is involved in these experiments. It has been shown here that both these haemolysins contribute to virulence. The principal rôle is played by α-haemolysin, which has a preponderating effect on the size of the lesions produced by the intramuscular inoculation of *Staph. pyogenes* in mice and is of importance in maintaining infection. The second haemolysin, which has been found in significant titres in a relatively small proportion of strains, is associated with an effect additive to that of α-haemolysin in determining the size of the lesions.

The results obtained here in coagulase tests on *Staph. pyogenes* with mouse

plasma confirm the finding of Field and Smith (1945) who obtained positive reactions with mouse plasma in the slide test, and those of Gorrill (1951) who was successful with both tube and slide tests. Our failure to find a significant correlation between virulence and the results of the tube test is probably associated with the weak and late reactions obtained with mouse plasma in a test which is subject to interference by a number of factors, which may include fibrinolytic activity. On the other hand, the slide test, which gives immediate reactions that are associated with virulence to the mouse, has the advantage of being subject to fewer variables than the tube test, and probably yields a better estimate of the amount of coagulase capable of clotting mouse plasma.

The general picture that emerges from these experiments in mice is consistent with what is to be expected from the known activities of haemolysin, coagulase and fibrinolysin. The size of the lesion at one day after inoculation is mainly determined by damage to the tissues by the haemolytic toxins, of which α-haemolysin is the most important. A further contribution to the initial size of the lesion is made by coagulase, which probably inhibits the phagocytosis of staphylococci by altering their surface properties as suggested by Hale and Smith (1945), and so promotes invasion of the tissues by staphylococci. In the subsequent progress of the infection the most important factors detected here are the continuing effect of α-haemolysin and fibrinolysin, of which the latter would appear to facilitate further invasion of the staphylococci by breaking down fibrin barriers. Coagulase also enhances the progress of the infection, but only to the second day, after which coagulase determines a more rapid regression of the infection probably by promoting the formation of fibrin barriers, and so preventing further invasion of the tissues and favouring the defence mechanisms of the host.

Our findings on the effects of α-haemolysin and coagulase on virulence are in substantial agreement with those of other workers on experimental staphylococcal infections. They confirm the work of Burnet (1929) and subsequent authors on the importance of α-haemolysin in determining virulence, and give added precision to the correlation between α-haemolysin production and virulence found by Christie, North and Parkin (1946) in their experiments on the killing power of staphylococci in mice. Regarding the part played by coagulase in determining virulence, Smith, Hale and Smith (1947) have shown that certain staphylococcal strains which are capable of coagulating guinea-pig plasma are more virulent to guinea-pigs than other strains, and that the virulence of staphylococci to mice can be enhanced by suspending the organisms in coagulable plasma. On the basis of their results these authors suggest that coagulase is a major factor in the initiation and development of staphylococcal lesions, but make no claim that it is the only or even the most important factor. We have also found that coagulase contributes to the determination of virulence in mice, but only in so far as strong coagulase reactions in the slide test with mouse plasma are associated with an effect additive to the much more significant effect of α-haemolysin.

SUMMARY.

The virulence to mice of 75 strains of *Staph. pyogenes* has been assessed by measuring the amount of swelling produced in the thigh after intramuscular inoculation.

It has been shown that the most important of the *in vitro* properties tested in determining virulence to mice is α-haemolysin production.

Haemolytic activity against sheep red cells, coagulase production and fibrinolytic activity have been shown to be associated with effects on virulence additive to the effect of α-haemolysin.

No correlation has been found between virulence and hyaluronidase production, colour, gelatin liquefaction, mannitol fermentation or resistance to penicillin.

We wish to thank Mr. T. E. Cowan, F.C.I.S., F.R.S.S., Records Officer, Middlesex Hospital, for his help in this investigation, and for allowing us to quote his statement that "the graphs indicate correlation, which is confirmed by a statistical examination of the figures."

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THE ACTION OF MUCIN IN PROMOTING INFECTIONS: THE ANTICOMPLEMENTARY EFFECT OF MUCIN EXTRACTS AND CERTAIN OTHER SUBSTANCES.

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NUNGESTER, Wolf and Jourdonais (1932) and Miller (1933) found that the lethal dose of many bacteria in experimental infections could be lowered by injecting them with mucin. Since this discovery mucin has frequently been used as a method of promoting infection with organisms which are relatively avirulent for the common laboratory animals. A wide variety of views has been expressed about the way in which mucin produces this effect. Inhibition of leucocytosis in the peritoneal cavity (Ercoli, Lewis and Harker, 1945), damage to phagocytes (Miller and Castles, 1936; Maccabe and King, 1951), inhibition of phagocytosis by a coating effect on bacteria (Tunnicliff, 1940), and delay in the intraphagocytic digestion of bacteria (Nungester, Jourdonais and Wolf, 1936; McLeod, 1941) have all been reported. Other experiments have been done to show that mucin inhibits the bactericidal action of body fluids (Oerskov, 1940; Sindbjerg-Hansen, 1943), that it inhibits antibody uptake by organisms (Keefer and Spink, 1938; Olitzki, Shelubsky and Efrati, 1947), and that mucin has a growth-promoting effect on bacteria (McLeod, 1941). Changes in capillary permeability have also been postulated (Sandage and Stark, 1949; Elberg and Gale, 1949). The subject has been reviewed by Olitzki (1948), who concluded that most of the reported effects could be attributed to one basic feature, a coating effect of mucin on bacteria.

Most of the reported work has been done with commercial dried hog gastric mucin. This is a mixture of substances which gives an opaque, granular and highly viscous suspension. Different batches vary considerably in their activity. Recently Smith (1950*a*, *b*; 1951*a*, *b*), Smith, Harris-Smith and Stanley (1951) and Smith, Gallop and Stanley (1952) have fractionated gastric mucin, using an assay of activity based on the intraperitoneal injection of *Salm. typhi* in mice. They have shown that the effect of mucin is due to a synergic combination of two non-specific physical agencies, viscosity and particulate residue, with a more specific "third factor." The action of this third factor is only fully manifested when it is injected with particulate matter in a viscous medium. The third factor owes its activity to the presence of at least two carbohydrates. It has recently been proved that the most active of these, polysaccharide A, is a heparin (Smith, 1951*b*). This paper is mainly concerned with the properties of polysaccharide A. The non-specific physical factors and the other components of the third factor, which are important in contributing to the effect of mucin, are not discussed here.

This fractionation provided the opportunity to examine the actions of relatively potent resistance-lowering substances without the complications caused by using crude gastric mucin.

MATERIALS AND METHODS.

Mucin extracts.—The extracts used were prepared by Dr. H. Smith as described in his papers cited above. The earlier work was done with a fraction called tryptic mucin (Smith *et al.*, 1952). This material is a mixture of substances, but is free from particulate matter and gives a non-viscous solution.

Hydrolysed tryptic mucin was used as a control in many experiments. A batch of tryptic mucin was boiled for 2 hr. under reflux condenser with 2N HCl. The product was neutralised with 2N NaOH. Solutions were made up so that the final NaCl content was 0·85 per cent.

Three purified carbohydrate fractions, called polysaccharides A, B and C, were later prepared from tryptic mucin. Polysaccharide A was used in later experiments. After the identification of this substance as a heparin, commercial heparin (Evans, Lescher and Webb) was also used in many tests. Other substances which were examined are described in the course of the paper.

These substances were used in solution in physiological saline, after the pH had been adjusted to 7·2–7·4.

Haemolysis tests.—Blood for complement was obtained by heart puncture from large male guinea-pigs. The serum was separated and used as a source of complement. Red cells were used as a 3 per cent suspension in saline; they were prepared from defibrinated sheep blood by centrifugation and four washes in a large volume of saline. Haemolytic antibody was prepared in rabbits. The serum was stored in the cold room without added preservative: 1 minimal haemolytic dose (M.H.D.) was contained in 0·0006 ml. of this serum.

In titrations of anticomplementary action serial dilutions of complement were made up in 0·9 ml. When different substances were compared, the complement dilutions were prepared in an equal increment series, each tube having a serum concentration 80 per cent that of the previous tube.

To each tube was added 0·1 ml. of the test substance, or 0·1 ml. saline in the control series. The racks were kept at 37° for 1 hr. To each tube was added 0·5 ml. of 3 per cent sheep cells, and a preliminary reading was taken after a further 1 hr. incubation at 37°. The racks were left in the cold room over-night and a final reading taken on the following day. The usual controls were included in each test, and repeated in the presence of each test substance.

Tests of the bactericidal effect of serum.—Fresh guinea-pig serum was used in most of these experiments. Fresh rat serum was also used in a few tests. The principle of the test was to inoculate each tube with the same number of bacteria, and to do a plate count on suitable dilutions after 2 hr. incubation at 37°. The technique used was shown to give reproducible results when tested on replicate tubes, and the type of result obtained has been illustrated in Table IV. The control tube with normal serum showed the maximal killing of bacteria by the dilution of serum used. Another control tube contained serum which had been heated to 56° for 30 min., and the count on this tube showed the extent of bacterial growth in the absence of complement.

Each tube contained 0·8 ml. diluted serum, 0·1 ml. test solution or saline, and 0·1 ml. bacterial suspension. When different anticomplementary substances were being compared, duplicate tubes were put up for each concentration of each substance. The bacterial inoculum varied in different tests, but was usually *ca.* 10,000 bacilli.

The organism was *Salm. typhi*, strain Ty2. A suspension was dried in the gelatin-ascorbic acid medium described by Stamp (1947). Tubes were taken up in tryptic meat digest broth (T.M.B.) and sown on tryptic meat agar (T.M.A.) slopes. The slopes were incubated at 37° for 18 hr. and the growth washed off with phosphate buffer (pH 7·6). The suspension was made up to a standard opacity and suitable dilutions prepared in phosphate buffer.

Phagocytic tests.—Human blood was obtained by venepuncture and defibrinated with a bent glass rod. The blood was centrifuged at 1200–1500 r.p.m. for 5 min., and after col-

lecting the serum the deposit was re-suspended in Locke's solution and centrifuged again. This was done four times. The organism was *Staphylococcus aureus*, Oxford "H" strain, grown on T.M.A. slopes for 16–20 hr. at 37°. The growth was washed off in 5 ml. saline and transferred to a small flask containing 1–2 mm. glass beads. The flask was rotated by hand for 5 min. to produce a clump-free suspension. The suspension was made up to a standard opacity and diluted in saline. The test was done in paraffined tubes 5 × 1 cm. Into each was pipetted 0·25 ml. cell suspension, 0·25 ml. serum and 0·5 ml. bacterial suspension, made up in saline or in the test solution immediately before the experiment was set up. The tubes were fitted with paraffined corks and rotated end-over-end at 15 r.p.m. for 30 min. at 37°. Films were made, dried rapidly in a current of warm air, and stained with Leishman's stain. For each test system 100 polymorphonuclear leucocytes were counted on each of two slides, giving a total sample size of 200 polymorphs. The tables showing results of these tests indicate that fairly small differences in phagocytic rate may prove to be highly significant. This accuracy is achieved by attention to the details of technique and the use of a large sample size.

Anticoagulant assay.—Citrated sheep plasma was re-calcified in the presence of falling dilutions of the test substance, as described by Foster (1942).

Assay of infection-promoting activity in mice.—The revised technique has been described by Smith *et al.* (1951). Large groups of mice were injected intraperitoneally with *Salm. typhi*, together with the substance under test, 0·4 per cent charcoal and dilute tragacanth solution to a standard viscosity. Charcoal and tragacanth in the concentrations used were themselves inactive in promoting infection. The dose of *Salm. typhi* is 5000 organisms, 1000 times less than the LD50 of this strain for intraperitoneal injection in broth. Viable counts were done by the method of Miles, Misra, and Irwin (1938). The concentration of test substances used was, as far as possible, adjusted to give death rates of 20–80 per cent, and the most probable activities were calculated by probit analysis.

EXPERIMENTAL AND RESULTS.

The effect of tryptic mucin on sheep cell haemolysis.

Tryptic mucin was found to inhibit specific lysis of sheep red cells. The order of inhibition was such that a final concentration of 0·05 per cent mucin gave a five-fold diminution of complement titre. In preliminary experiments this inhibition was found both as a reduction of complement titre in the presence of a constant amount of antibody (5 M.H.D.) and as a reduction of antibody titre in the presence of a constant amount of complement (5 M.H.D.). The mechanism of this action was examined with the following results:

1. Tryptic mucin has an anticomplementary action. The inhibition of haemolysis was removed in the presence of excess complement. This was shown by estimating the degree of inhibition produced by a selected dilution of mucin in a complement titration. A titration of haemolytic antibody was then set up with the same mucin concentration, using an excess of complement relative to the titre in the presence of mucin. The usual inhibition of haemolysis no longer occurred (Table I).

2. In a titration with falling amounts of complement, an inhibitory action on cell sensitization might affect the haemolytic titre within the range over which antibody and complement are mutually replaceable. Tryptic mucin was examined to detect any interference with cell sensitization. Sheep cells were made up in a 3 per cent suspension containing 5 M.H.D. antibody and mucin to a final concentration of 0·5 per cent. The suspension was left at room temperature for 1 hr. and in the refrigerator over-night. Another suspension, which was made up without mucin, had the same treatment and was used as a control. Both suspensions were centrifuged and washed, and complement titrations were done using each group of cells. The titres were identical. This

TABLE I.—(a) *Complement Titrations, Showing Inhibition of Haemolysis after Preliminary Incubation of Complement Dilutions with Tryptic Mucin.*

Complement dilution	1:100								
Ml. Complement	0·1	0·2	0·3	0·4	0·5	0·6	0·7	0·8	0·9
A. Preliminary incubation without tryptic mucin	—	—	(±)	+	+++±	++++	++++	++++	++++
B. Preliminary incubation with 0·075 per cent tryptic mucin	—	—	—	—	—	—	—	—	—
C. Preliminary incubation with 0·025 per cent tryptic mucin	—	—	—	—	((±))	((±))	(±)	(±)	±

Complement dilution	1:10									Cell controls.		Complement controls.		
Ml. Complement	0·1 *	0·2	0·3	0·4	0·5	0·6	0·7	0·8	0·9	Sensitized.	Unsensitized	1:10	1:20	1:100
A. Preliminary incubation without tryptic mucin	++++	++++	++++	++++	++++	++++	++++	++++	++++	—	—	—	—	—
B. Preliminary incubation with 0·075 per cent tryptic mucin	—	(±)	++	+++±	++++	++++	++++	++++	++++	—	—	—	—	—
C. Preliminary incubation with 0·025 per cent tryptic mucin	+±	+++±	++++	++++	++++	++++	++++	++++	++++					

Readings after standing in cold room over night.

(b) *Antibody Titrations, Showing Failure of Tryptic Mucin to Lower Titre in the Presence of Excess Complement.*

Ml. Haemolytic antibody 1:500	0·1	0·2	0·3	0·4	0·5	0·6	0·7	0·8	Controls. Complement only.	Cells only.	Antibody only.	Mucin + complement. No serum.
Titration in the absence of tryptic mucin, using 3·3 M.H.D. complement relative to complement titration A*	+	+++±	++++	++++	++++	++++	++++	++++				
Titration in the presence of 0·025 per cent tryptic mucin, using 3·3 M.H.D. complement relative to complement titration C†	+	+++	++++	++++	++++	++++	++++	++++	—	—	—	—

Relative excess of complement calculated with reference to the preliminary readings of the complement titrations given above.

* Preliminary reading of A showed a titre of 1:140, so that 3·3 M.H.D. was given by 1:42 complement.

† Preliminary reading of C showed a titre of 1:33, so that 3·3 M.H.D. was given by 1:10 complement.

((±)) = Faint trace of haemolysis, with varying degrees of haemolysis to ++++ = complete haemolysis.

experiment showed that tryptic mucin did not interfere with cell sensitization in the presence of excess antibody. A similar experiment using groups of cells sensitized with varying amounts of antibody showed that even slight degrees of sensitization were not inhibited by mucin.

The inhibitory effect of tryptic mucin was repeatedly shown to occur in the absence of free antibody, the degree of inhibition being the same whether the sensitized cells used were washed or unwashed.

3. Tryptic mucin did not act on previously sensitized cells in any way which prevented their lysis by complement. Cells were sensitized with 5 M.H.D. antibody, centrifuged and washed free of excess antibody. Mucin was added to the suspension to a final concentration of 0·5 per cent. The suspension was left on the bench for 1 hr. and in the refrigerator over-night. It was then centrifuged, washed and centrifuged again. These cells, made up in saline to a 3 per cent suspension, were used in a complement titration. The titre was the same as that of a control series.

4. Tryptic mucin might still combine with red cells, though the tests described show that it does not do so in preference to antibody nor in a way which inhibits the action of complement on sensitized cells. The supernatant fluid (S.N.F.) from the centrifuged suspension described in paragraph 3, which had been made up to contain 0·5 per cent mucin, was used in a complement titration, and its inhibitory effect was compared with that of a 0·5 per cent solution of mucin which had not been in contact with red cells. A 1:4 dilution of the S.N.F. was also compared in its anticomplementary action with a 0·125 per cent solution of mucin which had not been in contact with red cells. Each pair of titrations gave identical readings, indicating that mucin had not been adsorbed on to red cells.

5. The mucin extract had no effect on the fragility of sheep cells in hypotonic saline. The extract has a negligible salt content and the pH of solutions was always adjusted to 7·2 to 7·4.

6. Hydrolysed tryptic mucin was tested as an anticomplementary agent. It had no effect in some experiments and produced a negligible degree of inhibition in others.

It was concluded that tryptic mucin inhibits haemolysis by an anticomplementary action, and that no other component of the system is affected.

The effect of tryptic mucin on the bactericidal action of serum.

The anticomplementary action of this extract was also shown by an inhibition of the killing of *Salm. typhi* by fresh guinea-pig or rat serum. Since tryptic mucin is a mixture containing much peptide material, it was arguable that the inhibition was simply due to the presence of an added supply of nutrients in an unfavourable environment. This possibility was excluded when it was found in repeated experiments that hydrolysed tryptic mucin had no inhibitory effect on the bactericidal power of serum (Table II).

The anticomplementary action of more highly refined mucin fractions.

While this work was in progress Dr. Smith was separating more active resistance-lowering fractions from tryptic mucin, and three polysaccharides, called A, B and C, were finally extracted. Many tests were done with mucin

TABLE II.—*Inhibition of Killing of* Salm. typhi *by Guinea-pig Serum, using Tryptic Mucin (T.M.).*

	No. of viable bacteria/ml. 2 hr. after inoculation.
Normal serum 1:4	1,600
„ „ + 0·075 per cent T.M.	8,800
„ „ „ „ Hydrolysed T.M.	900
„ „ + 0·025 „ T.M.	5,200
„ „ „ „ Hydrolysed T.M.	900
„ „ + 0·005 „ T.M.	3,800
„ „ „ „ Hydrolysed T.M.	1,000
Heated serum 1:4	16,500

fractions at different stages of extraction, using both the haemolytic and bactericidal techniques. A number of points emerged from this work :

1. Of the fractions derived from tryptic mucin, polysaccharide A had the most powerful anticomplementary action. Polysaccharide B, which is contaminated with a proportion of A, had a slight anticomplementary effect.

2. No other mucin fraction derived from tryptic mucin had any appreciable anticomplementary effect. These points are illustrated in Table III.

TABLE III.—*Anticomplementary Effect of Different Mucin Fractions.*

	Complement dilution at titre.
Control	1:120
Tryptic mucin, 0·05 per cent	1:42
Polysaccharide A, „ „	1:17
„ B, „ „	1:85
„ C, „ „	1:120

Thus the anticomplementary action which had been detected and analysed in tryptic mucin was probably entirely due to the polysaccharide A component. With the identification of polysaccharide A as a pure heparin (Smith, 1951*b*), this substance was compared with a commercial heparin (Evans) as an anticomplementary agent. In the haemolytic test heparin (Evans) was distinctly more inhibitory than polysaccharide A. In the bactericidal test no difference could be detected between the degrees of inhibition produced by the two substances (Table IV).

TABLE IV.—*Inhibition of Bactericidal Effect of Serum by Polysaccharide A and Heparin (Evans).*

	No. of viable bacteria/ml. 2 hr. after inoculation.
Normal serum 1:2·5	1,700
„ „ + Polysaccharide A 0·05 per cent	8,500*
„ „ „ 0·025 „	7,750*
„ „ + Heparin (Evans) 0·05 „	9,150*
„ „ „ 0·025 „	7,150*
Heated serum 1:2·5	15,400

* Mean of duplicate tubes.

Since it had now been shown that the most important factor in the resistance-lowering activity of tryptic mucin was its heparin component, the remaining

work on mucin fractions described here was done with heparin. In the rest of this paper the word " heparin " refers either to polysaccharide A or to heparin (Evans); in the tables of experimental results the source of the heparin is indicated. All the experiments were done using polysaccharide A, and many of them were also confirmed using heparin (Evans).

The opsonic action of mouse serum.

The killing of *Salm. typhi*, strain Ty2, by guinea-pig or rat serum has no immediate relevance to infection in the mouse, since mouse serum is lacking in whole complement (Brown, 1943), and we have confirmed that it does not kill these organisms. The rôle of mouse serum in a phagocytic system was therefore examined. A highly artificial system, consisting of thoroughly washed human cells, mouse serum and staphylococci, was necessary to enable quantitative tests to be done. The mouse serum was separated from the pooled defibrinated blood of many mice bled by heart puncture immediately after breathing had stopped under ether anaesthesia.

In this system it was found that—

(*a*) Fresh mouse serum promoted the phagocytosis of staphylococci. The action was well marked but inferior to that of human serum.

(*b*) This action was inhibited by heparin.

(*c*) Mouse serum heated to 56° for 30 min. was devoid of opsonic activity.

In Table V a representative test is shown.

TABLE V.—*Opsonic Action of Mouse Serum: Effect of Heating Serum, and of Adding Polysaccharide A.*

Medium.	No. of bacteria in 100 polymorphs.		Mean No. of bacteria per polymorph.	Difference between means.	*t*.
	Slide I.	Slide II.			
Mouse serum	855	787	8·21	4·35	8·6
Mouse serum + poly A, 0·1 per cent	395	378	3·86		
Heated mouse serum	30	54	0·42		
Locke's solution	33	32	0·32		

t = Student's " *t* " test.

The mode of inhibition of phagocytosis by heparin.

The depression of phagocytosis by heparin has been described by several authors (Allgöwer, 1947). The inhibition is slight with ordinary anticoagulant concentrations of heparin, and phagocytic tests are frequently done in the presence of heparin as an anticoagulant. We have examined the mode of action of heparin in a phagocytic system consisting of human cells, human serum and staphylococci, and have found that the inhibition is due solely to the anticomplementary action of this substance. The evidence for this statement is summarized below:

1. Heparin had no effect on the rate of bacterial multiplication under the conditions of the phagocytic test. Staphylococci were grown in normal serum with and without heparin under conditions identical to those of the phagocytic test, i.e., rotation of tubes for 30 min. at 37°. The presence of heparin did not affect the final count.

2. Heparin had no effect on the total leucocyte count under the conditions

of the phagocytic test. Agglutination of leucocytes by heparin has been observed in blood which has been left standing for several hours (Wilander, 1938) but this did not occur in the conditions described.

3. Heparin inhibited phagocytosis when fresh serum was present in the system (Table VIA).

TABLE VI.—*Effect of Polysaccharide A on Phagocytosis.*

A. In presence of complement.

Medium.	No. of bacteria in 100 polymorphs. Slide I.	Slide II.	Mean No. of bacteria per polymorph.	Difference between means.	t.
Fresh human serum only . . .	1198	1140	11·69	3·55	6·29
Fresh human serum + poly A, 0·1 per cent	825	803	8·14		

B. In absence of complement.

Heated human serum only . . .	709	722	7·15	0	—
Heated human serum + poly A, 0·1 per cent	641	790	7·15		

4. Heparin produced no inhibition of phagocytosis in the absence of complement. Heated human serum has some opsonic effect for staphylococci; the effect is smaller than that induced by fresh serum, so that a larger bacterial inoculum is necessary to give an adequate number of ingested cocci in the control series. In experiments using washed human cells, heated human serum, and staphylococci, heparin failed to inhibit phagocytosis. In Table VIB examples are given of this type of test. This result also implies that no other component of the phagocytic system is affected by heparin, but more direct tests were done to detect any action of heparin on phagocytes.

5. Washed blood cells were mixed with heparin in Locke's solution and incubated for 30 min. The cells were washed three times in Locke's solution, and then put up in a standard phagocytic test with fresh human serum and staphylococci. The phagocytic rates were the same as those of control series.

A comparison of various anticomplementary compounds.

When it was found (Smith, 1951*b*) that the component of the "third factor" of mucin most active in lowering resistance to peritoneal infection was a heparin, there were at least two striking effects to be considered as the possible cause of this action, the anticoagulant and the anticomplementary effects. There is a general relationship between blood-clotting and complement action, and many substances are known which inhibit both processes. The two inhibitory effects do not, however, run parallel in their intensity. A series of experiments was therefore carried out in which various substances were assayed for anticoagulant, anticomplementary and resistance-lowering activity. A preparation of heparin (Evans) was used as a standard in all the tests. This was compared with sodium polyanethol sulphonate (Liquoid-Roche), a sulphated dextran, a specimen of which was kindly sent by Dr. W. T. J. Morgan, and chlorazol fast pink (Gurr), an anticoagulant dye. These substances were compared in six types of experiment:

(*a*) Anticoagulant assay *in vitro*.

(*b*) Anticoagulant activity in mice. Each substance was injected intraperitoneally in mice under the conditions of the assay of resistance-lowering activity, i.e., in combination with 0·4 per cent charcoal and dilute tragacanth to standard viscosity. One hour later the coagulation time of the blood was measured by the Dale-Laidlaw method (Figure).

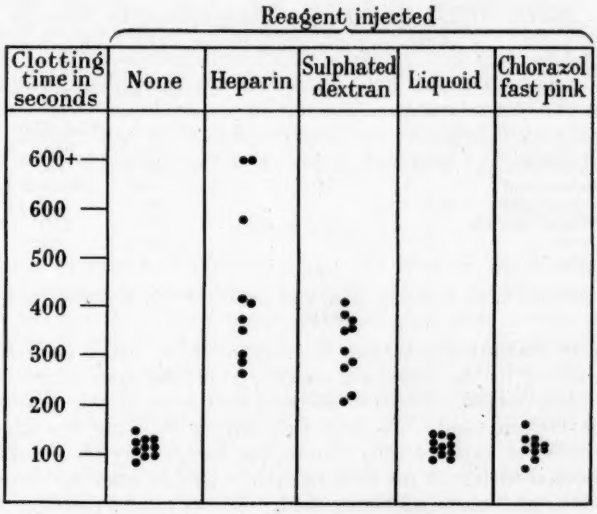

FIG.—Clotting times in mice 1 hr. after intraperitoneal injection of 0·5 ml. of an 0·05 per cent solution of the following reagents with charcoal and tragacanth.

TABLE VII.—*Inhibition of Bactericidal Effect of Guinea-pig Serum by Various Anticomplementary Substances.*

Test substance.	No. of viable bacteria/ml. 2 hr. after inoculation with *Salm. typhi*. (Mean of duplicate tubes for test substances.)
Normal serum 1:4	660
„ „ + Heparin 0·025 per cent	6,700
„ „ + Liquoid „ „	14,700
„ „ + Sulphated dextran „ „	4,700
„ „ + Chlorazol pink „ „	2,600
„ „ + Heparin 0·0125 per cent	4,700
„ „ + Liquoid „ „	9,700
„ „ + Sulphated dextran „ „	1,360
„ „ + Chlorazol pink „ „	660
Heated serum 1:4	20,200

(*c*) Inhibitory effect on sheep cell haemolysis. The usual procedure of the test was changed in order to make a quantitative comparison between the test

substances. Falling amounts of the substances were incubated with a constant amount of complement. The end-point was taken as the concentration of the material which just failed to inhibit haemolysis by 3 M.H.D. of complement.

(*d*) Inhibitory effect on the bactericidal action of guinea-pig serum. An example of this type of test is given in Table VII.

(*e*) Effect on the phagocytosis of staphylococci (Table VIII).

TABLE VIII.—*Inhibition of Phagocytosis by Various Anticomplementary Substances.*

Test substance.	No. of bacteria per 100 polymorphs.		Mean No. of bacteria per polymorph.
	Slide I.	Slide II.	
Serum	1014	1006	10·1
„ + Heparin (Evans) 0·05 per cent	447	430	4·4
„ + Chlorazol pink „ „	36	58	0·5
„ + Chlorazol pink 0·01 „	494	496	4·9
„ + Sulphated dextran „ „	323	290	3·1
„ + Liquoid „ „	16	14	0·15
Locke's solution	4	2	0·03

Comparing chlorazol pink, 0·01 per cent, with sulphated dextran, 0·01 per cent; $t = 5$.

(*f*) Assay of resistance-lowering activity in mice, using each test substance as the third factor in the standard assay. This was done three times and the results have been pooled. Controls showed that none of the materials was toxic in the concentrations used. To give an example showing the effect of heparin on infection by *Salm. typhi* in mice, one of the assays may be described. Twenty mice were injected with 0·05 per cent heparin together with the standard charcoal and tragacanth, and none of these died. Of 80 mice receiving 5000 bacteria with charcoal, tragacanth, and various concentrations of heparin from 0·05 per cent to 0·00625 per cent, 35 died; in the absence of heparin the death-rate was 1/10. The bacterial controls, receiving 5×10^6 bacteria in broth, showed a death rate of 5/10.

TABLE IX.—*Summary of Tests of Anticomplementary, Anticoagulant, and Resistance-lowering Activities of Various Substances.*

	Relative potency in promoting infection in mice. (Figures in brackets are fiducial limits for P = 0·95.)	Anti-coagulant assay *in vitro* (International units).	Anti-coagulant action in mice (see figure).	Inhibition of bactericidal effect of serum (Table VIII.)	Inhibition of phagocytosis. (Table IX.)	Inhibition of haemolysis (arbitrary units).
Heparin (Evans)	1	85	++	+++	+	16
Liquoid	1·1 (0·6–2·1)	4·5	0	++++	++++	100
Sulphated dextran	0·71 (0·38–1·3)	14	+	++	+++	33
Chlorazol fast pink	0·18 (0·09–0·35)	0·8	0	+	++	27

In Table IX the results of these varied experiments are summarized. It is apparent that all these substances, when injected with particulate matter in a viscous medium, promote infection with *Salm. typhi* in the mouse. Liquoid, heparin and the sulphated dextran have about the same effect, with the most probable activities in the order just given. Chlorazol pink has a significantly

weaker action than the other substances. The anticoagulant tests show that liquoid, which is as active as heparin in the mouse assay, is 20 times weaker as an anticoagulant; the coagulation time of mouse blood is not prolonged 1 hr. after the intraperitoneal injection of liquoid in the highest concentration used (0·05 per cent) in the mouse assay. The sulphated dextran is a powerful anticoagulant, only 5–6 times weaker than heparin. Chlorazol pink, on the other hand, is 100 times weaker than heparin.

The anticomplementary tests are difficult to assess because the relative activity of the substances is not the same in all the systems. Liquoid is the most powerfully anticomplementary substance by any test, and the sulphated dextran is always more strongly inhibitory than chlorazol pink. Heparin produces the least marked inhibition of the four substances in tests of haemolysis or of phagocytosis; but in the bactericidal test the inhibitory effect of heparin is constantly and definitely greater than that produced by the sulphated dextran or by chlorazol pink.

DISCUSSION

Much of the diversity of opinion about the way in which mucin promotes infection is certainly due to the technical difficulties of working with suspensions of crude gastric mucin. The present experiments, using non-particulate non-viscous extracts of mucin, throw some light on previous work on the effect of crude mucin in serum reactions. Keefer and Spink (1938) and Pacheco and Péres (1940) have found that the inhibitory effect produced by mucin in bactericidal tests varies with the concentration of antibody; and they conclude that mucin inhibits the action of antibody on bacteria. If a substance reduces the complement level to the range within which antibody and complement are mutually replaceable, an inhibitory effect varying with the antibody concentration will indeed be found. Pacheco and Péres did not carry out complement titrations, and Keefer and Spink reported that crude mucin failed to inhibit sheep cell haemolysis. An inhibition of haemolysis might well be difficult to detect in the presence of crude gastric mucin, which forms an opaque white suspension; and if a change in haemolytic titre had been masked in this way, the results in the antibody titrations could have been due to anticomplementary action. This argument could not, of course, be applied to the inhibition of typhoid "O" agglutination by mucin reported by Olitzki *et al.* (1947), but we were unable to confirm their observation, either with Wilson granular mucin or with tryptic mucin. Although it is difficult to do an accurate agglutination test in the presence of granular mucin, mucin granules can be distinguished from bacteria under the microscope; and using this method we could detect no inhibition of agglutination by mucin.

The inhibition of haemolysis in the presence of heparin is well known, but a possible action on other components of the system has not often been considered. Ecker and Gross (1929) showed clearly that heparin is anticomplementary, but they could not exclude a supplementary action on haemolytic antibody. The experiments described show that the anticomplementary action of tryptic mucin, which is due to its heparin content, is unassociated with a subsidiary action on antibody or any other component.

Does anticomplementary activity contribute to the resistance-lowering effect of mucin? Several workers have stated that mucin inhibits extra-cellular

lysis of bacteria in the peritoneal cavity, and an anticomplementary action could well account for these findings. The resistance-lowering effect of mucin has, however, often been reported for organisms which are insusceptible to bacteriolysis by serum (Olitzki, 1948). The present experiments on mouse serum show that, despite its failure to kill bacilli, this serum can opsonize at least one species of organism, and that this effect is heat labile and is inhibited by heparin. Delay in intraphagocytic digestion in the presence of mucin has been observed by Nungester *et al.* (1936) and by McLeod (1941), and there is reason to believe that complement is necessary for the killing of bacteria within phagocytes (Robertson and Sia, 1927). Again, Delaunay and Pagès (1946) state that complement plays an important part in the physiology of the leucocyte. It is thus possible to ascribe inhibition of bacteriolysis and the various anti-phagocytic actions to a common cause: anticomplementary activity. We have shown that the anticomplementary action of tryptic mucin is accounted for by its heparin content, and the evidence given suggests that this action may be a factor in the resistance-lowering effect of crude mucin. The methods of sterilization used by many workers would not destroy any heparin contained in the specimen. On the other hand, it is difficult to relate *in vitro* tests to processes of infection, and little is known about the way in which typhoid bacilli are killed in the mouse. The importance of anticomplementary action in accounting for the resistance-lowering effect of heparin is therefore, at this stage, impossible to assess. The rôle of heparin in natural infections is quite unknown.

The comparison of substances with varying anticomplementary and anticoagulant activities (Table IX) suggests that anticoagulant action is not the main cause of the infection-promoting effect of these substances. This action may, however, be a subsidiary cause, and a possible mechanism is suggested by the work of Wood, Smith and Watson (1946) on surface phagocytosis. These authors have shown that fully virulent bacteria which are not ingested by phagocytes in the ordinary type of *in vitro* test in the absence of antibody, can be ingested when trapped against a suitable surface or between cells. Smith and Wood (1949) have observed that this process occurs on strands of fibrin. If the injection of an anticoagulant delayed or reduced surface phagocytosis by inhibiting fibrin formation, an infection-promoting effect might easily result. Work is now in progress with the object of testing some of the possibilities which have been discussed.

SUMMARY.

Tryptic mucin, an impure extract of gastric mucin, inhibits sheep cell haemolysis. This is due to an anticomplementary action and no other component of the system is affected.

Tryptic mucin also inhibits the killing of *Salm. typhi* by guinea-pig or rat serum.

The anticomplementary action of tryptic mucin is due to the heparin which it contains.

Mouse serum has an opsonic action, which is heat-labile and is inhibited by heparin.

The inhibition of phagocytosis by heparin is due to its anticomplementary action. The depression of bacteriolysis and of various phagocytic functions may be ascribed to a common cause—anticomplementary action.

A number of substances with anticoagulant and anticomplementary properties have been compared. They all have resistance-lowering activity when injected into mice with particulate matter in a viscous medium.

The possible rôles of anticomplementary and anticoagulant effects in promoting infection are discussed.

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INVESTIGATION INTO THE PRODUCTION OF BACTERIOSTATIC SUBSTANCES BY FUNGI. PRELIMINARY EXAMINATION OF THE TWELFTH 100 SPECIES, ALL BASIDIOMYCETES.

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THIS paper, the twelfth in the above series, is the eighth dealing exclusively with Basidiomycetes. The fungi were isolated by ourselves as indicated by the letter W before the culture number. For several years only potato dextrose and malt were used for the liquid medium on which the fungi were grown in order to produce metabolism solution. In this work, however, following certain rather promising experiments, a medium consisting of 5 per cent malt extract plus 1 per cent peptone, referred to as "peptone malt," was used. Whereas the time period of growth on the medium was originally 30 days, and later raised to 45 days, in the present series the metabolism solution was tested every 5 days for a period of 60 days, because it was felt that, within limits, the antibacterial activity of the metabolism solution increased in proportion to the length of growing time. The test was by the "Wilkins-Revised" method (Wilkins, 1949) against the two representative types *Bacterium coli* and *Staphylococcus aureus* as before. As in previous papers the results are arranged in three classes, "strongly positive," "weakly positive" and "negative," but only the first class is dealt with in detail.

LIST OF FUNGI EXAMINED.

Strongly Positive.

	Result of test on liquid medium.			
			Zone diam. (mm.)	
	Time in days.	Medium.*	*Bact. coli.*	*Staph. aureus.*
Clitocybe obsoleta (Batsch) Fr. W654A . .	40	PD	+	25
C. rivulosa (Pers.) Fr. W6183A . . .	35	PD	28	19
Clitopilus prunulus (Scop.) Fr. W666A . .	30	M	+	27
Coprinus picaceus (Bull.) Fr. W616D. . .	45	PM	28	25
C. picaceus (Bull.) Fr. W616E	50	PM	18	22
Pleurotus palmatus (Bull.) Quél. W6158A . .	35	M	28	22
Ptychogaster albus Cda. W6191A . . .	35	M	15	24

* M = malt; PD = potato dextrose; PM = peptone malt.

Weakly Positive.

Boletus edulis (Bull.) Fr. W6185A; *Clitocybe aurantiaca* (Wulf.) Studer W682D, W682E and W682G; *Collybia butyracea* (Bull.) Fr. W610E; *Cyathus*

striatus (Huds.) Pers. W639B ; *Laccaria laccata* (Scop.) B. and Br. W6161B ; *Lentinus vulpinus* (Sow.) Fr. W6119A ; *Lepiota naucina* Fr. W6166A ; *Marasmius confluens* (Pers.) Karst. W683B ; *M. peronatus* (Bolt.) Fr. W613D ; *Merulius lacrymans* (Wulf.) Fr. W6198A ; *Polyporus betulinus* (Bull.) Fr. W620G ; *P. fragilis* Fr. W6193A and W6193B ; *P. Schweinitzii* Fr. W6145B ; *Psalliota xanthoderma* Genev. var. *lepiotoides* R. Maire W680A ; *Stereum hirsutum* (Willd.) Fr. W6112B ; *Tricholoma nudum* (Bull.) Fr. W61H and W61J.

Negative.

Amanita pantherina (D.C.) Fr. W6199A ; *Armillaria mellea* (Nahl.) Fr. W688D ; *Boletus bovinus* (Linn.) Fr. W6178A ; *B. elegans* (Schum.) Fr. W6150A and W6150B ; *Bovista plumbea* Fr. W6189A ; *Clavaria botrytis* (Pers.) Fr. W6159A ; *Clitocybe geotropa* (Bull.) Fr. W614C and W614D ; *C. nebularis* (Batsch) Fr. W644C ; *Collybia distorta* Fr. W6181A ; *C. dryophila* (Bull.) Fr. W6163A ; *C. fusipes* (Bull.) Berk. W662C ; *C. radicata* (Relh.) Berk. W69C ; *Coprinus comatus* (Fl. Dan.) Fr. W623D and W623E ; *C. micaceus* (Bull.) Fr. W6110D ; *Cortinarius pholideus* Fr. W6154A ; *Crucibulum vulgare* Tul. W638B ; *Cyathus olla* (Batsch) Pers. W6184A ; *Flammula sapinea* Fr. W624D ; *Fomes pomaceus* (Pers.) Big. and Guill. W6180A and W6180B ; *F. ulmarius* (Sow.) Fr. W6127A ; *Ganoderma applanatum* (Pers.) Pat. W6103C ; *Hydnum coralloides* (Scop.) Fr. W6202A ; *Hypholoma sublateritium* (Schaeff.) Fr. W6174A ; *Laccaria laccata* (Scop.) B. and Br. W6161A ; *Lenzites betulina* (Linn.) Fr. W6190A ; *Lepiota excoriata* (Schaeff.) Fr. W6187A ; *L. mastoidea* Fr. W6182A ; *L. procera* (Scop.) Fr. W625C ; *L. rhacodes* (Vitt.) Fr. W615D ; *Lycoperdon giganteum* (Batsch) Pers. W6160A ; *L. perlatum* Pers. W695D ; *Mutinus caninus* (Huds.) Fr. W6195A ; *Mycena epipterygia* (Scop.) Fr. W612E and W612F ; *M. galericulata* (Scop.) Fr. W626D ; *M. polygramma* (Bull.) Fr. W611C ; *M. sanguinolenta* (A. and S.) Fr. W6196A ; *Omphalia umbilicata* (Schaeff.) Fr. W676A ; *Panus stipticus* (Bull.) Fr. W647B ; *Phallus impudicus* (Linn.) Pers. W657B ; *Pholiota aurivella* (Batsch) Fr. W658B ; *P. spectabilis* Fr. W6144B ; *P. squarrosa* (Müll.) Fr. W6169B ; *Pleurotus sapidus* Schulz W664C ; *P. ostreatus* (Jacq.) Fr. W6201A ; *Polyporus adustus* (Wild.) Fr. W629C ; *P. brumalis* (Pers.) Fr. W6200A ; *P. chioneus* Fr. W6203A ; *P. dryadeus* (Pers.) Fr. W6139B ; *P. giganteus* (Pers.) Fr. W6124A ; *P. squamosus* (Huds.) Fr. W6121B ; *P. stipticus* (Pers.) Fr. W6129B ; *Polystictus albidus* Massee W6153A ; *P. hirsutus* (Wulf.) Fr. W6171A ; *Psalliota amethystina* Quél. W684B ; *P. augusta* Fr. W622C ; *P. campestris* (Linn.) Fr. W6137A and W6137B ; *P. haemorrhoidaria* Kalchbr. W671C ; *P. sylvatica* (Schaeff.) Fr. W669B ; *P. sylvicola* (Witt.) Fr. W667E ; *P. villatica* (Brond.) Magn. W673H ; *P. xanthoderma* Genev. W677J ; *Psilocybe polytrichi* Fr. W7197A ; *Stropharia aeruginosa* (Curt.) Fr. W628D ; *S. semiglobata* (Batsch) Fr. W665B ; *Trametes rubescens* (A. & S.) Fr. W6136A ; *Tricholma flavobrunneum* Fr. W6120A ; *Tubaria furfuracea* (Pers.) W. G. Sm. W6192A.

The results follow the trend of the last two papers (Wilkins and Partridge, 1950 ; Wilkins, 1952) in having a relatively low, 27 per cent, proportion of positive isolates. One explanation of this is that in all these cases the isolations were kept for some months without subculturing. While there is no direct experimental evidence to prove the point, it is my opinion that many fungi gradually lose their antibacterial activity when in culture. This is particularly so on certain media,

if cultures are kept on the same medium all the time or if not subcultured fairly frequently. Unfortunately this does not explain the relatively high, 50 per cent, positive results of cultures which were obtained from other workers and institutions, whose cultural history was not known, but which, in some cases, had not been recently subcultured.

Of the fungi here tested for 60 days instead of the normal 45 days, only three showed an increase of zone size during the last 15 days. The 45-day period was therefore tentatively regarded as adequate. Keeping a culture which is growing in 25 ml. of medium, and tested every five days, free from contamination for six weeks is in itself a problem, and unnecessary lengthening of the growing period is to be avoided.

Before critical comment is made the peptone malt medium is being further tested.

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THE DISPOSAL AND ORGAN-DISTRIBUTION OF RADIO-IODINATED BOVINE SERUM PROTEINS IN CONTROL AND SPECIFICALLY-SENSITIZED RABBITS.

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THE site of formation of antibodies and the dependence of the process on the presence and persistence of the operative antigen have been studied extensively during the past half century. Most of the earlier attempts to trace the fate of a foreign soluble protein after injection have depended upon some prior labelling which enabled its subsequent distribution in the tissues to be followed by either chemical or histological methods. In experiments of the former kind, various metals, notably arsenic, in aromatic complexes have been attached to carrier proteins by diazotisation and other means; in the latter, distinctive dyes have been similarly coupled. Since radioactive isotopes have become widely available for immunological studies, however, there has been an increasing tendency to substitute these tracer elements for foreign metals and dyes. This is partly for the technical reason that radioactive isotopes can often be readily identified and estimated subsequently, and partly for the theoretical one that the attachment of large substituent dye and metal-carrying aromatic compounds may affect significantly the ultimate distribution of the antigenic complex in the tissues (Dixon, Bukantz and Dammin, 1951; Dixon and Talmage, 1951; Gitlin, Latta, Batchelor and Janeway, 1951). Of the radioactive elements available, sulphur, phosphorus and iodine are all suitable for such purposes; iodine, however, has particular advantages because its penetrating γ-radiation enables its location in the tissues to be readily ascertained in the still living animal.

The present work on the disposal of foreign antigens was carried out with bovine serum albumin and bovine serum γ-globulin, both of which had been lightly labelled with ^{131}I. With these marked proteins it has proved practicable to compare the fates of typical soluble antigens for the first 48 hours after their intravenous injection into normal and specifically-sensitized rabbits. The present paper is concerned with the immunological aspects of this work; its metabolic findings will be described later (Laws, 1952).

MATERIALS AND METHODS.

Animals.

All the observations were made on full-grown albino rabbits weighing between 2 and 3 kg. In most of the experiments, to ensure better comparability, normal and sensitized rabbits were paired for weight just before the injection of the antigen. For the collection of urine, the rabbits were kept for the duration of the experiment in metabolism cages. In a few instances, to obtain urine specimens shortly after the injection, the rabbits were catheterized.

Antigens.

Bovine serum albumin ("Armour") was mainly used as the carrier protein. This preparation is stated to contain at least 95 per cent of albumin, and is a convenient and effective antigen. In a few experiments, bovine serum γ-globulin ("Armour") was used; by electrophoretic analysis this preparation is 98–100 per cent globulin.

Radio-iodination of the antigen.

Radioactive iodine was obtained as carrier-free sodium iodide. Iodination of the proteins was performed by the trace-labelling method of Wormall and his colleagues (Banks, Francis, Mulligan and Wormall, 1951; Francis, Mulligan and Wormall, 1951). The albumin or globulin was dissolved initially in water or saline respectively to form a 7·5 per cent solution; later, during the process of iodination, such solutions became diluted to about 3 per cent. In most of the experiments 0·2 ml. of the initial solution of the protein was treated with 0·02 ml. of 5N NH_4OH. The radioactive iodide solution (250 or 500 μC) as obtained from Harwell was added to 0·2 ml. of an I_2-KI mixture in water which contained 1·23 mg. I_2 and 1·57 mg. KI per ml. After it had stood for a few minutes, this latter solution was added to the ammoniated protein, and the mixture, after standing for an hour at room temperature, was dialysed at 0° in Visking transparent cellulose tubing against frequent changes of water or saline to free it from uncombined iodide and iodine. At the completion of dialysis the protein was in equilibrium with a dialysate that contained no more than 30 c.p.m. per 10 ml. when counted in the standard type M 6 liquid counter. This residual uncombined radioactive iodine was insufficient to cause any appreciable error in the subsequent counts on blood and other materials.

Dosage of radioactive antigen.

The amount of radio-iodinated antigen injected into the rabbits was based on the amount of protein present in the preparation rather than on its radioactivity, which varied with the amount of ^{131}I used. The initial protein solution contained 15 mg. of albumin or globulin, and in most of the experiments this was ultimately divided equally between a pair of rabbits, one of which was normal and the other specifically sensitized. Each animal thus received intravenously about 7 mg. of the protein if allowance is made for slight losses in the preparation. It is noteworthy that these doses are very much smaller than those employed by other workers (Latta, 1951; Gitlin *et al.*, 1951), who have used amounts of protein that were several hundred times as great. The effect that such gross differences in dosage may have on the subsequent distribution and breakdown of the radioactive antigen in the tissues will be considered in the discussion.

Sensitization of rabbits.

The sensitizing antigen was an iodinated albumin or globulin prepared by the same technique as the radio-iodinated protein except that only non-radioactive iodine was used. The sensitizing and test antigens were thus chemically identical. Sensitization was effected by intraperitoneal injections of 30 mg. of this antigen at weekly intervals; animals were used 2–4 weeks after the final injection. Although no quantitative precipitin reactions were carried out, the sera from several of the rabbits gave positive qualitative tests for the presence of antibodies to the iodoproteins.

Exsanguination.

All rabbits were finally bled out by dividing their carotid arteries under anaesthesia.

Estimation of radioactivity in blood, urine and tissue samples.

During the earlier part of the work counts were made with a GM 4 tube and a lead castle arranged for β-particle counting on dried material. Subsequently, facilities for γ-counting with G 10 lead cathode tubes became available. This latter type of counter was used in two ways. Firstly, a well-shielded, collimated tube was used for external counts on selected portions of the living animals. This tube was also used for preliminary work on the γ-counting of specimens of blood and urine. Latterly it became possible to increase the efficiency of this form of recording by using an apparatus in which a pair of these lead cathode

tubes, wired in parallel, were mounted side by side in a lead block. The material to be counted was contained in a standard flat-sided glass cell placed in a hole in the block between the two tubes: it thus lay close to the sides of both cathodes. Since much greater volumes of blood and urine can be used effectively for γ-ray counting because of the absence of internal absorption in the specimen, the sensitivity of this latter arrangement proved comparable with that of the β-counter. On account of its greater ease of use and its lesser variability in recording, this twin-tube apparatus was employed in all the later work.

With β-particle counting on blood, urine and tissues, self-absorption within the sample is inevitable. For blood, whose composition is relatively constant, the preparation of the samples by the following technique ensured satisfactory comparability in their counts. Citrated blood, in 0·1 ml. amounts, was placed in aluminium foil dishes; after lysing and spreading with 0·4 ml. water, the films were dried under an electric lamp bulb. With urine, whose composition is less uniform, errors from self-absorption in dried samples are greater. Nevertheless, similarly dried thin films prepared from 0·5 ml. centrifuged urine enabled information to be gained as to the comparative rates of excretion of radioactive iodine in normal and sensitized rabbits. With tissues, the amount of radioactivity was too small for any similar thin dried film technique to be practicable. It was possible, however, to obtain mutually comparable counts on different tissues by using them in thicker layers, so that, as a result of the progressive self-absorption in the deeper parts, the recorded counts for β-radiation soon approached asymptotic values (see Tubiana, 1950). Although the greater amount of tissue necessarily present in such thick films resulted in a corresponding increase in the γ-radiation that reached the counter, the efficiency of the GM 4 tube for this type of radiation is so low that, within the range of values observed, no significant error was introduced. This point is discussed by Latta (1951), who reached a similar conclusion. In practice about 0·5 tissue brei was spread smoothly in an aluminium foil dish and counted while still moist.

In the later experiments, counts on blood, urine and tissues were made with the twin-tube γ-counter. Since this apparatus permitted the recording of the total γ-counts on the antigen solution injected and on the various samples obtained subsequently, it was possible to construct a rough balance-sheet for the disposal of the radio-iodinated protein and its degradation products. In practice γ-counts were made on 2 ml. blood, 5 ml. urine and 2 g. tissue, except for the spleen, lymph nodes and thyroid, for which whole organs were used.

RESULTS.

Blood.

The comparative radioactivities of the blood samples obtained from associated pairs of normal control and sensitized rabbits after injection with radioactive proteins are set out in Table I. Throughout this table the counts are expressed as percentages of the initial values obtained from the control member of the pair, i.e., of the level reached a few minutes after injection, when mixing in the circulating blood may be assumed to be complete (see Reeve, 1947–48). This mode of expressing the results was adopted largely because the counts obtained on the blood samples of the sensitized animals fell so rapidly from the moment of injection that for these rabbits it was not possible to obtain any stable initial value.

From Table I it can be seen that in the control rabbits, the values of the radioactivity of the blood following injection of the labelled protein show two phases of decrement. In the first, which lasts from 5 to 10 hr., there is a sharp drop from the initial count. In the second the decline is roughly exponential, the rate of loss appearing to depend on the concentration remaining. With a survival period of 48 hr. it is not possible to evaluate this rate of loss. A few animals were allowed to survive for 72 hr., and the counts obtained from them suggest that the " half-life " of the radio-iodinated albumin in the body is about five days. This estimate is in agreement with that of Knox and Endicott (1950).

TABLE I.—*Changes in the Radioactivity of the Blood of Control and Sensitized Rabbits, Expressed as Percentages of the Initial Counts in the Former, at Various Intervals after the Intravenous Injection of Radio-Iodinated Bovine Serum Albumin and Serum Globulin.*

Rabbit number.	Number of sensitizing injections.	Radioactivity after various intervals.				
		Initial.	5 hr.	24 hr.	48 hr.	72 hr.
Rabbits injected with albumin.						
34	—	100	87	43	31	—
33	3	4·7	9·2	2·8	2·4	—
35	—	100	50	30	18	—
36	4	10·1	5·4	2·0	1·2	—
38	—	100	64	34	30	—
37	1	42·2	12·4	2·2	0·9	—
40	—	100	63	39	31	25
39	4	95	8·5	3·0	1·6	2·1
42	—	100	62	52	22	—
41	2	5·5	5·3	5·6	0·8	—
45	—	100	—	49	32	—
43	4	77	—	11·4	8·6	—
44	3	85	—	16·5	11·1	—
48	—	100	81	—	—	—
39	5*	80	12·1	—	—	—
43	5*	43	7·4	—	—	—
Rabbits injected with globulin.						
47	—	100	63	43	50	—
46	2	74	9·5	3·5	6·9	—

* The previous injection of radioactive albumin had been given 6 weeks before; the earlier findings are recorded above.

In the control animals the radioactivity of the blood is almost wholly attributable to radioactive iodine that is still united to substances in colloidal form, for only 2 to 3 per cent of the total activity is capable of removal by dialysis.

In the sensitized rabbits the radioactivity of the blood fell much more sharply than in the comparable control animals. After 5 hr. about two-thirds of the radioactive protein was still present in the circulating blood of the control animals; in that of the sensitized rabbits at this time less than one-tenth remained. In the latter animals the first phase in the fall is particularly difficult to demonstrate, because a large proportion of the injected material was usually removed from the circulation within the first few minutes.

In those sensitized rabbits in which the blood radioactivity fell especially rapidly during the first few minutes, it was noted that the counts declined no

further during the remainder of the first 24 hr., and sometimes even rose slightly. Dialysis of such bloods showed that up to one-half of their activity arose from radioactive iodine in a dialysable form; it seemed that much of the enhanced rate of removal of the antigen from the circulation was accompanied by some degradation of the protein that led to the liberation of radioactive compounds of small molecular size. In all the sensitized animals the radioactivity of the blood was hardly detectable at the end of 48 hr.; this finding is to be contrasted with the persistence of nearly 30 per cent of the initial counts in the blood of the controls. In a single pair of rabbits in which radio-iodinated globulin was used, a similar contrast was observed in the rates of disappearance of radioactivity from the blood.

Urine.

The comparative excretions of radioactive iodine in control and sensitized rabbits have been followed both quantitatively and qualitatively. Qualitatively, the iodine present in the urine is exclusively in a dialysable form. Through the use of paper chromatography, it has been shown to occur in two fractions:

TABLE II.—*Excretion of Radioactive Compounds in the Urine of Control and Sensitized Rabbits after the Intravenous Injection of Radio-Iodinated Bovine Serum Albumin.*

Rabbit number.	Number of sensitizing injections.	Daily excretion (as percentages of the inoculated doses).			
		0–24 hr.	24–48 hr.	Total for 48 hr.	
				Control.	Sensitized.
		Estimations made by β-counter.			
34	—	7·1	3·7	10·8	—
33	3	40·0	7·1	—	47·1
35	—	11·7	3·8	15·5	—
38	—	1·6	2·1	3·7	—
37	1	7·1	28·0	—	35·1
40	—	1·1	2·2	3·3	—
39	4	42·0	9·0	—	51·0
42	—	4·7	0·6	5·3	—
41	2	13·9	5·1	—	19·0
		Estimations made by γ-counter.			
40	—	1·4	4·4	5·8	—
39	4	65·4	32·3	—	97·7
42	—	9·2	1·8	11·0	—
41	2	25·1	13·2	—	38·3
47*	—	16·9	13·1	30·0	—

* This rabbit was injected with radio-iodinated bovine γ-globulin.

inorganic iodide and an iodinated amino-acid, almost certainly 3:5-diiodotyrosine, in an organic complex hydrolysable by acid. These observations will be recorded more fully elsewhere (Laws, 1952). The quantitative findings for the urinary excretion of the radioactive iodine as a whole are set out in Table II.

In control rabbits some excretion of radioactive iodine begins within the first few minutes of the injection of the protein. At first the amounts are small, but within an hour or two the rate increases. Usually the total amount excreted during the first 24 hr. exceeds that in any subsequent similar period—a finding which is probably to be accounted for by the progressive fall in the concentration of the antigen in the blood. In 16 control rabbits the mean excretions for the first and second 24-hr. periods, as estimated by β-counting were 10·5 and 4·6 per cent respectively of the amount injected. The values for urinary excretion, however, show a wide scatter, which is probably largely attributable to the gross individual variations in the volumes of urine voided. When the amount is small, the total excretion of radioactive iodine is also generally low, though its concentration may be raised. It seems that when the urinary output is small, a larger proportion of the radioactive iodine becomes fixed in the thyroid gland. In the clearing of the blood of diffusible radioactive iodine, fixation in the thyroid and excretion by the kidney seem to be to some extent complementary.

In the sensitized rabbits urinary excretion of the radioactive iodine proceeds much more rapidly than in the respective controls. Both by γ-counting and by the less complete β-counting, the amount passed in the urine in the 48-hr. period is 4–5 times greater in the former. In sensitized rabbits a peak in the excretion rate is often reached at the end of 5–6 hr., and in most of them from a quarter to one-half of the injected iodine is lost in the first 24 hr. In one control and two sensitized rabbits that were killed at 5 hr., and whose results are consequently

TABLE III.—*Radioactivity of Various Organs of Control and Sensitized Rabbits after the Intravenous Injection of Radio-Iodinated Bovine Serum Albumin.*

Rabbit number.	Sensitized or control.	Type of recording.	Radioactivity (c.p.m.). Spleen.	Liver.	Kidney.	Muscle.	Thyroid.
Animals killed at 48 hr.							
34	C	β-counting	40	42	99	6	3,942
33	S	,,	10	6	7	2	13,012
35	C	,,	125	101	293	49	34,596
36	S	,,	207	23	18	0	22,330
38	C	,,	19	19	48	0	1,896
37	S	,,	8	5	13	0	4,684
42	C	,,	51	74	104	22	4,086
41	S	,,	38	7	21	0	5,918
42	C	γ-counting*	10	1,160	201	9	1,854
41	S	,,	9	216	40	0	2,941
Animals killed at 5 hr.							
48	C	γ-counting*	10	1,320	378	1	187
39	S	,,	22	1,872	248	6	3,691
43	S	,,	44	546	180	1	3,465

* γ-counts are expressed as totals for the whole organ except for muscle, where it is given as per g.

not included in Table II, the excretions were 4·2, 38·3 and 60·2 per cent respectively. In one sensitized rabbit, which was observed for 72 hr., a further 5 per cent was present in the urine of the third 24-hr. period. The number of the sensitizing doses of antigen given previously did not seem markedly to affect the excretion rate or the total excreted in 48 hr.; too few rabbits were used, however, for any definite conclusion on this point.

Tissues.

Because the liver and spleen contain so large a proportion of the reticulo-endothelial system, particular attention was paid to the comparative radioactivities of these organs in control and sensitized animals. It can be seen from Table III, in which these results are set out, that the counts obtained on samples of liver taken from sensitized animals after death 48 hr. after injection were much lower than those obtained from their respective controls. Similar differences (see Table IV) were found when external counts were made with a collimated

TABLE IV.—*Radioactivity over Certain Areas of the Body, Measured with a Collimated γ-Counter, in Control and Sensitized Rabbits at Various Intervals after the Intravenous Injection of Radio-Iodinated Bovine Serum Albumin.*

Rabbit number.	Sensitized or control.	Radioactivity (c.p.m.) over stated area. Liver. 5 hr.	Liver. 24 hr.	Liver. 48 hr.	Chest. 5 hr.	Chest. 24 hr.	Chest. 48 hr.	Thyroid. 5 hr.	Thyroid. 24 hr.	Thyroid. 48 hr.
35	C	365	274	212	387	287	197	316	1142	1372
36	S	1145	63	15	199	61	13	133	847	918
38	C	—	50	27	—	60	58	—	94	91
37	S	—	38	6	—	27	14	—	319	207
40	C	114	112	104	182	143	114	86	189	319
39	S	110	58	16	79	38	0	95	179	210
42	C	141	121	85	171	151	112	116	386	265
41	S	251	27	25	106	24	2	33	285	269
48*	C	136	—	—	208	—	—	139	—	—
39*	S	102	—	—	96	—	—	438	—	—
43*	S	62	—	—	60	—	—	298	—	—

* Rabbits killed at 5 hr.

TABLE V.—*Radioactivity over the Liver Area, Measured with a Collimated γ-Counter in Control and Sensitized Rabbits at Various Intervals after the Intravenous Injection of Radio-Iodinated Bovine Serum Albumin.*

Rabbit number.	Sensitized or control.	Radioactivity (c.p.m.). 1–2 hr.	5 hr.	6 hr.	24 hr.
35	C	483	365	349	274
36	S	1763	1145	511	63
48*	C	243	136	—	—
39*	S	466	102	—	—
43*	S	354	62	—	—

* Rabbits killed at 5 hr.

γ-counter over the liver area just before death. In several rabbits this latter procedure was also used to determine the changes in the radioactivity of this organ within the first few hours after injection ; the results are shown in Table V. At the end of the first hour liver area counts were higher in the sensitized than in the control animals. Since by this time the radioactivity of the blood of the former had fallen well below that of the latter, this higher count could not have resulted from the blood present in the organ, but must be attributed to a local concentration due to some specific cause. By 5 hr. the inequality was less evident, and in the single pair observed at 24 hr., the liver area count of the sensitized rabbit was much below that of its respective control.

The spleen is too small and too variable in position for its radioactivity to be estimated during life. In sensitized rabbits killed at 5 hr. the radioactivity of this organ was greater than that of the controls, in spite of the higher blood radioactivity of the latter. After 48 hr. the values for sensitized rabbits were mostly lower than in the corresponding controls, though this difference is less clear than with the liver. The total radioactivity of the spleen is always much smaller than that of the liver, as can be seen for the γ-counts for the whole organs which are set out in Table III.

The counts obtained with muscle give some indication of the distribution of radioactivity in the extra-cellular fluids : in all instances in which it was measurable at 48 hr. it was lower in the sensitized animals.

The counts observed for the thyroid gland and kidneys are probably less related to the distribution of the antigenic protein in the body than to the products of its destruction. With one exception the thyroid counts are higher in the sensitized rabbits, when made both at 5 and at 48 hr. The kidney counts, on the other hand, are always lower in the sensitized than in the control animals ; this might be expected from the finding that the radioactivity of the urine of the former falls away more quickly than that of the latter—the peak of excretion being higher and earlier.

DISCUSSION.

The introduction of radio-iodinated proteins into immunology has obviated some of the difficulties inseparable from the use of synthetic antigens marked with metals or dyes. When the protein is treated by the method originally devised by Wormall (see Francis *et al.*, 1951), the iodine becomes substituted in the tyrosine, and once incorporated, it shows no tendency, within the time limits and under the physiological conditions of such experiments, to exchange significantly with any surrounding free iodine (Eisen and Keston, 1949). It is liberated only when the protein carrier itself is broken down, for as Knox and Endicott (1950) have shown, after the intravenous injection of radio-iodinated bovine serum albumin into rabbits, estimates of the persistence of the antigen in the circulation which are derived from measurements of its radioactivity are closely paralleled by those obtained from quantitative specific precipitin reactions. Moreover, iodine as a label has particular advantages for investigating the differentiating features of the " primary " and " secondary response," as in the present work, for the marker introduced with the primary and subsequent inoculations, though *chemically* and *biologically* identical, can readily be rendered *physically* distinguishable by substituting for the final inoculations the radioactive ^{131}I for

the earlier non-radioactive ^{127}I. This physical distinguishability permits a quantitative study to be readily made of the differences in the disposal of marked antigens in specifically-sensitized and control animals.

From the experiments described above it can be seen that control and specifically-sensitized rabbits dispose of intravenously-injected iodinated proteins at very different rates. This is well shown by the comparative rates of decline in the radioactivities of the respective blood samples. With the same dosage of inoculum, the values in the sensitized animals were always lower than in their paired controls. Even when the sampling was carried out only 3 minutes after the inoculation, the difference was already appreciable, and it became progressively larger in succeeding hours. After 48 hours the radioactivity of the blood of the controls was still about 25 per cent of its initial value (a finding which is very close to that of Knox and Endicott (1950) for the same antigen), whereas that of the sensitized rabbits was by this time scarcely measurable. It is evident, therefore, that sensitization has led to the development of a mechanism which promotes the rapid removal of the foreign protein from the circulating blood.

It seems possible that this clearance mechanism, very evident with low dosages of antigen, may become overstrained when large amounts of foreign protein are injected intravenously. As has been pointed out above, our animals received only a few mg. of radio-iodinated protein, whereas the doses given by other workers have almost invariably been very much larger (Dixon *et al.*, 1951; Dixon and Talmage, 1951; Gitlin *et al.*, 1951; Latta, 1951). It is conceivable that such large inocula may temporarily strain the capacity of the clearance mechanism, and obscure the more rapid falls in the blood values seen with lower dosages.

The current belief that much of any intravenously-injected foreign antigen is removed in the liver is supported by estimates of the comparative radioactivities of organs that were made either by external regional counting during life or by tissue sample counting after death. In the sensitized rabbits, external counts suggest that during the first few hours radioactive substances accumulate rapidly in the hepatic region. By the end of 24 hours—sooner in some animals—this localized concentration disappears, for much of the radioactive iodine has by this time been excreted. In the control animals, on the other hand, no distinctively high concentration of radioactivity over the hepatic region was observed at any period. In these animals the counts over the chest area remain consistently higher than those over the liver—a finding which suggests that the radioactivity of both regions is mainly due to the blood contained in the underlying structures. It is noteworthy that the fall in the count over the liver area in these control rabbits is slow, and parallels the fall in the radioactivity of their blood.

From the data for the urinary excretion of ^{131}I, it appears that once the antigen has been removed from the blood, it is katabolized rapidly. Though any estimate of excretion rates in rabbits can only be rough because of dilution with residual bladder urine, the present findings clearly show that there is no selective retention of the antigen in the tissues of the sensitized animals; in fact the injected iodinated protein is destroyed substantially faster in these than in the controls. Whether the intracellular destruction of the antigen proceeds more rapidly in sensitized rabbits is uncertain; it would seem that the increase in their rate of excretion of ^{131}I could be sufficiently accounted for by more rapid clearance of the blood.

It was hoped that the experiments described above might throw light on the mechanism of the secondary response that follows the injection of a soluble antigen into a specifically-sensitized animal. It was provisionally supposed that a significant difference between sensitized and unsensitized animals might take the form of a greater retention of any injected antigen by the tissues of the former. For this supposition these experiments provide no support. The dialysable nature of the radioactive products excreted in the urines of both the control and the sensitized rabbits shows that, with or without specific immunity, there is no tendency for even this small-sized foreign protein to be excreted unchanged. In both groups of animals this iodinated albumin is disintegrated in the tissues—in those of the sensitized even more rapidly than those of the controls. How this accelerated reaction could stimulate a more profuse liberation of antibodies into the blood of sensitized animals remains obscure. It is difficult to believe that the more rapid fixation and destruction of the antigen could act simply by providing additional templates for the elaboration of specific antibody. Moreover, the finding that in sensitized rabbits the antigen is largely katabolized, and its disintegration products excreted within two or three days, is difficult to reconcile with the common experience that in the secondary response the antibody titre only begins to rise towards the end of this period, and continues to rise for several days afterwards. It seems that the effect of sensitization in promoting the more effective and prolonged formation of specific antibody must be sought, as Burnet and Fenner (1949) supposed, in some induced, long-lasting modification of the cells of the antibody-forming apparatus, and not in a persistence of the operative antigen itself.

SUMMARY.

A comparison has been made of the disposal of soluble antigens by specifically-sensitized and normal control rabbits; radio-iodinated bovine serum albumin and bovine serum γ-globulin were used.

Following their intravenous injection both these proteins disappeared from the blood in sensitized animals more quickly than in the corresponding controls. In both groups much of the circulating foreign protein appeared to have been removed by the liver. The excretion of radioactive iodine, in the form of dialysable compounds in the urine, also proceeded more rapidly in the sensitized rabbits.

The prompter excretion of radioactive degradation products in the urines of the sensitized animals indicates that the secondary response characteristic of immunization is not associated with any prolongation of the retention time of the antigen in the tissues.

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THE DEGRADATION OF PROTEINS LABELLED WITH RADIOACTIVE IODINE IN CONTROL AND SENSITIZED RABBITS.

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A COMPARATIVE study of the rates of disposal and the tissue distribution of foreign serum proteins labelled with radioactive iodine in control and specifically-sensitized rabbits has been made by Laws and Payling Wright (1952). These experiments offered an opportunity for investigating the katabolic breakdown of these proteins in so far as this is revealed by the radioactive excretory products detectable in the urine. Hitherto, such products have not received detailed consideration. According to Knox and Endicott (1950), both Dixon and his associates and Williams have found inorganic iodides and diiodotyrosine to be the chief radioactive degradation products excreted. In preliminary communications, the present author (Laws, 1951*a* and *b*) has pointed out that the organic component is probably more complex. The present paper deals with this point more fully, and also presents some data on the relative proportions of inorganic and organic iodine present in such urines.

MATERIALS AND METHODS.

Details of the methods used for the iodination of the proteins and the sensitization of the rabbits have been given earlier (Laws and Payling Wright, 1952), and will only be summarized here.

Most of the experiments were performed with bovine serum albumin (Armour) iodinated by the method of Wormall and his colleagues; in a few, bovine serum γ-globulin ("Armour") was used. Following dialysis in Visking transparent cellulose tubing, the solution of the iodinated protein (whose iodine content was less than 1 per cent) was divided into two parts; these were injected intravenously into paired control and specifically-sensitized rabbits. The animals were kept in metabolism cages and their urines collected under toluene; any ammoniacal specimens were discarded. Urines for assay were collected at 24 and 48 hr. after injection; with sensitized rabbits whose rate of excretion of ^{131}I-containing compounds was rapid, specimens were obtained more frequently.

Treatment of urine specimens.

Urines were dialysed in transparent cellulose sacs of the same type as those used for the original protein preparation. Any urinary radioactive products capable of passing through the cellulose membrane must therefore have arisen through a breakdown of the injected protein. After centrifugation, protein in the urine was precipitated by addition of an equal volume of 30 per cent trichloroacetic acid. After standing at room temperature for 30 min., the specimen was centrifuged until clear. The radioactivities of the supernatant and of the original urine diluted to the same extent with water, were then compared.

Chromatography.

Paper chromatography with Whatman 3 MM paper was used for the separation of the radioactive constituents of the urine. The amount used for each run was based on the initial radioactivity of the specimen, since several hundred c.p.m. over the initial spot were

needed to obtain a chromatogram that would demonstrate the separation and location of the radioactivity. In most runs, 0·3 to 0·4 ml. of centrifuged, but otherwise untreated, urine were used. Since salts and urea cause some blurring of the final chromatograms, an attempt was made to remove them with urease and electrolytic de-salting in an apparatus with a carbon anode and a mercury cathode. Unfortunately, this treatment failed, for the hydrogen produced at the cathode led to the displacement of the organically-bound iodine, so that all the radioactivity of the specimen was lost.

For the chromatography, three solvents proved satisfactory: phenol saturated with water, lutidine saturated with water, and an *n*-butanol-acetic acid mixture. Both one-way and two-way papers were used; in the latter separations, phenol was usually followed by *n*-butanol-acetic acid mixture. After having been run overnight, the papers were dried and developed with ninhydrin and heat. The radioactivity of the different sections of the paper was determined with a GM 4 β-particle counter enclosed in a lead shield and applied directly to its surface. On account of its ease and rapidity, this method is well suited for the identification of active sites, and as a guide when elution is to be carried out on the duplicate chromatogram.

Elution of selected areas of chromatograms was effected by slow percolation of water through the paper. When so passed, over a period of 2 to 3 hr., 3 to 5 ml. usually sufficed to remove the radioactive material. Hydrolysis of such eluates was effected by adding an equal volume of concentrated HCl and heating to 120° for 1 hr. in a sealed tube. The contents were then evaporated to dryness on a water bath to remove the HCl, and the product dissolved in water.

RESULTS.

1. *Qualitative.*

No qualitative difference was detected in the radioactive excretion products from the control rabbits and those previously sensitized to iodinated proteins. Dialysis of the urines of both groups of rabbits against water led quickly to an equilibrium of concentration of radioactivity inside and outside the sac. Repeated dialysis of the same urine specimen until its radioactivity was too small to be measured accurately still showed such an equilibrium—an observation which excludes the possibility that the urinary activity is associated with the excretion of any of the original injected protein unchanged. This is confirmed by the finding that the removal of all the trichloroacetic acid-precipitable material in the urine leads to no reduction in radioactivity.

For preliminary paper chromatographic analysis, phenol proved to be the most useful solvent, since it gave a sharp separation between the inorganic iodides, which moved little, and the more mobile organic radioactive constituents. With urines from rabbits which had received albumin, scanning of the paper with a GM 4 counter showed that the greatest radioactivity was invariably close to the starting point (RF 0·1) and resulted from inorganic iodides. The second peak of activity was fairly constantly associated with a spot (RF 0·5–0·6) that on development with ninhydrin failed to correspond with any mauve stain such as those produced by most amino acids. Commonly, 3 or 4 faint mauve areas were present, but the radioactive peak usually corresponded with a quite distinctive pale yellow spot. If parallel chromatograms on such urines were run, with and without the addition of tyrosine or radioactive 3:5-diiodotyrosine, it was found, both by ninhydrin development and by scaling for radioactivity, that these two substances ran further up the paper than the unknown radioactive excretory product. This strengthened the impression that the radioactive urinary constituent was not simple diiodotyrosine as some earlier workers had supposed.

Two-way chromatography in phenol followed by *n*-butanol-acetic acid mixture gave a further separation, the radioactive spot commonly forming a clearly-defined oval 1–1·5 cm. in diameter. In this solvent mixture the urinary constituent had an RF of 0·75, while tyrosine and 3:5-diiodotyrosine had values of 0·5–0·6. Inorganic iodide also had an RF of 0·5 in this mixture, but any confusion in the two-way chromatograms could be prevented by preliminary separation in phenol. In the centre of these papers ninhydrin development commonly disclosed three or four mauve areas, while the yellow spot seen in the one-way chromatograms appeared to run evenly to form a yellow streak with an RF of 0·75. Whether this coloured material was itself responsible for the radioactivity was not established. Chromatographic analysis of the urines of both the albumin- and globulin-injected rabbits showed closely similar distributions of activity. With the globulin animals, however, the second radioactive peak was rather higher in the phenol paper (RF 0·75), but with two-way chromatography in *n*-butanol-acetic acid mixture, the RF of this spot (0·75) was the same with both series of rabbits.

Elution of the organic radioactive material was performed on both one-way and two-way chromatograms with similar results. The eluted material was hydrolysed with hydrochloric acid and the residue, after removal of the acid, was run on a further paper ; as a control, an unhydrolysed eluate from another chromatogram was used. One-way secondary chromatograms showed that the position of the radioactive spot had been altered by the hydrolysis, and now coincided with that found for the 3:5-diiodotyrosine tracer. Ninhydrin development of these chromatograms revealed a number of mauve spots instead of the single yellow one typical of the unhydrolysed eluate. The results were similar when the hydrolysed products were run in any of the solvents used, though the positions of the radioactive spots naturally differed with each. Lutidine gave a particularly good separation of the unhydrolysed eluate and its radioactive hydrolysis product, since the former had an RF of 0·5, and both the latter and 3:5-diiodotyrosine had values of 0·8.

Two-way chromatography of the hydrolysate in phenol followed by *n*-butanol-acetic acid mixture gave a good separation of its constituent amino acids, but radioactive areas were usually difficult to detect on these papers. Comparisons with tracers of known amino acids run simultaneously showed the presence in the hydrolysate of glycine, lysine, leucine, arginine, tyrosine, valine and glutamic acid ; in addition, one spot remained unidentified. Control elutions of the areas in the original two-way chromatograms that lay immediately round the radicactive spot gave no evidence of the presence of these amino acids, so that they must presumably be regarded as constituents of the radioactive substance excreted.

2. *Quantitative.*

The total urinary excretion of radioactive substances following the intravenous injection of radio-iodinated serum albumin and γ-globulin has been recorded elsewhere (Laws and Payling Wright, 1952) ; the difference in the partition of this radioactivity between inorganic and organic iodine compounds will be considered here.

One-way paper chromatograms in phenol generally present two well-separated

and defined areas of radioactivity, and thus provide the means for estimating the ratio of the inorganic and organic iodine fractions. Though internal absorption in the paper causes a marked reduction in the radioactivity measurable with a β-recording G.M. counter, there is no reason to suppose that this will affect one fraction more than the other. Accordingly, counts were made of the two areas in chromatograms of urines from control and sensitized rabbits which had been injected with radio-iodinated protein. Only those chromatograms that showed good localization of activity were used. The results of counts on 8 specimens of urine from 4 control rabbits and on 16 specimens from 6 sensitized rabbits are given in Table I.

TABLE I.—*Comparison of Radioactivities of the Inorganic Iodide and the Organic Iodine Fractions of the Urinary Excretion Products of Sensitized and Control Rabbits following the Intravenous Injection of Radio-Iodinated Foreign Proteins.*

Rabbits.	Number of urine specimens examined.	Mean counts per minute.	
		Iodide spot.	Organic iodine spot.
Control (4) . .	8 .	62	41
Sensitized (6) . .	16 .	116	39

The means of the percentages of the total iodine excreted that was present in the form of iodide in the urines of the control and sensitized rabbits were $61{\cdot}6 \pm 2{\cdot}3$ and $72{\cdot}3 \pm 3{\cdot}1$ respectively. This finding indicates that sensitized rabbits eliminate a larger proportion of this radioactive iodine in the form of inorganic iodide, an alteration which is possibly attributable to a more complete breakdown of the foreign protein in the tissues of these animals.

DISCUSSION.

Earlier work by the present author (Laws, 1951*a*; Laws and Payling Wright, 1952) had suggested that the breakdown products of iodinated foreign proteins found in the urine included complex organic substances, and chromatographic analysis of these compounds was in progress when Knox and Endicott (1950) published the suggestion that inorganic iodide and 3:5-diiodotyrosine were the main substances concerned. Chromatography soon confirmed the presence of inorganic iodide, and there seems little doubt that this is quantitatively the chief end product of the katabolism of iodinated proteins. The chromatographic behaviour of the organic radioactive iodine compound, however, differed significantly from that of 3:5-diiodotyrosine. Several features pointed to its being some peptide derived from the partial degradation of the protein, and the evidence obtained from the study of its acid hydrolysates now sustains this belief.

The excretion of this peptide suggests that such foreign proteins are metabolised with difficulty, possibly owing to their configuration differing materially from those native to the rabbit. If this be so, the differences between the proportions of the iodine excreted as iodide and as an organic form may assume significance. From the greater proportion found as iodide in the urines of sensitized rabbits, it would seem that such animals have at least partially overcome the difficulty of breaking down the peptide in question. Perhaps the adaptation of enzymes to foreign substrates which this would entail is analogous to the adaptation of

formative enzymes postulated by Burnet and Fenner (1949) in their theory of antibody formation. With the present evidence, however, the possibility cannot be excluded that the increase in the ratio of the inorganic idodide to the iodinated peptide in the urines of sensitized rabbits is due to some alteration of the activity of the thyroid gland that would differentially affect the iodide fraction. There is as yet no evidence for such an alteration, but it cannot be excluded until similar experiments have been carried out in which the participation of the thyroid gland has been eliminated in some way.

SUMMARY.

It has been shown by chromatography that both control and specifically-sensitized rabbits injected intravenously with a radio-iodinated foreign protein excrete the iodine in the urine in two forms: the greater part is present as inorganic iodide, the rest is organically-bound, presumably to a peptide. On hydrolysis of the radioactive organic component a mixture of amino acids is produced, one of which behaves chromatographically like 3:5-diiodotyrosine.

In specifically-sensitized rabbits the ratio of iodide to organically-bound iodine in the urine following injection with radio-iodinated foreign protein is significantly higher than in normal control animals.

I wish to express my thanks to Dr. R. W. R. Baker for his valuable assistance in preparing and interpreting the chromatograms.

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INTRACELLULAR GROWTH OF PHAGES T1 AND T2.

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RECOGNISING the need for a new and more direct approach to the problem of how phage multiplies inside bacteria, Doerman (1948*a*) introduced the cyanide lysis technique to disrupt bacteria in which phage was multiplying. In that way it was possible to follow the intracellular growth of phage from the time of the addition of phage until natural lysis. The method is however an indirect one ; it relies on cyanide sharply arresting phage growth, after which lysis is effected by " lysis from without " due to a large excess of phage of another type. A method which does not depend on the action of some chemical to stop phage growth, was used by Anderson and Doerman (1952) who disrupted bacteria infected with phage by subjecting them to sonic vibrations. This technique yielded results similar to those obtained with the cyanide lysis technique, but is only applicable to those phages stable to the treatment, e.g., T3 and T7 (Anderson, Boggs and Winters, 1948), the larger *coli*-phages being themselves damaged by sonic vibrations.

In this paper a simple method is described for disrupting *Bacterium coli* by mechanical means, and is applied to some problems connected with the multiplication of T1 and T2.

METHODS.

Strain B of *Bact. coli* and the two phages used were obtained through the courtesy of Brigadier J. S. K. Boyd, F.R.S. A synthetic medium (SM) of the following composition was used : glucose 0·5 per cent, potassium hydrogen phosphate 0·9 per cent, ammonium chloride 0·05 per cent, ammonium sulphate 0·05 per cent, magnesium sulphate 0·001 per cent (0·00008 M), neutralised to pH 7·6 with sodium hydroxide. All chemicals were Analytical Reagent grade, dissolved in glass distilled water.

The phosphate buffer used in certain experiments was made by mixing equimolar solutions of disodium hydrogen phosphate and potassium dihydrogen phosphate in the ratio of 4:1. The pH was 7·6.

Stock phage T1 was made by infecting bacteria growing logarithmically in SM supplemented with 0·001 M magnesium at a multiplicity of 1 when the cell concentration was 4×10^8/ml. The titre of the lysate was then usually about 5×10^{10} phage/ml. Calcium, strontium and manganese could be substituted for the supplement of magnesium.

T2 was made similarly. There was no divalent metal requirement. The culture was allowed to lyse overnight and lysates contained around 10^{11} phage/ml.

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All phages were assayed by the double agar layer technique of Hershey, Kalmanson and Bronfenbrenner (1943), using 1 per cent peptone and 1 per cent agar.

RESULTS.

Disrupting the Bacteria.

Adequate provision has to be made in experiments on intracellular phage growth to ensure that all bacteria are infected at approximately the same time. The general practice was to concentrate bacteria in the log phase of growth about twenty times by centrifuging (using as the re-suspending fluid the growth medium rather than new medium to obviate a subsequent lag phase), and to add a suspension of phage to give the required multiplicity. The mixture was shaken by hand in a water bath at 37° for about one minute, then chilled, and centrifuged and washed *in the cold*. The cells were re-suspended at the original concentration in synthetic medium containing 0·05 per cent gelatin to protect the phage during the subsequent shaking treatment. This procedure yielded a population of bacteria all infected within one minute of each other and obviated centrifuging each sample, since there is *no* phage in the supernatant until the end of the latent period.

The infected bacteria were then incubated at 37°. At various times samples were withdrawn and the bacteria disrupted. This was done in the following manner.

Infected culture in 10 ml. aliquots was pipetted into cooled one-ounce screw-capped bottles containing 6 g. of ballotini glass beads (grade 14). The bottles were kept in an alcohol-solid carbon dioxide mixture until the samples began to freeze, generally after 10 to 15 seconds, and then transferred to an ice-salt mixture at 0°, where they were stored until all samples had been collected. They were then transferred to a special wooden box capable of holding 18 bottles and packed in ice. This box was then shaken in a vertical mechanical shaker at a rate of 350 oscillations per min. of an amplitude of 3 in. (7·2 cm.) After 20 minutes' shaking, by which time over 95 per cent of the bacteria had been disrupted, the amount of phage liberated from the bacteria was estimated.

The advantages of the method are (*a*) its great simplicity, no centrifuging or handling of the infected bacteria being required, (*b*) the certainty that all phage growth is arrested within 15 seconds of taking the sample, and (*c*) the fact that all samples are treated identically.

Intracellular Growth of T1.

Since the latent period of T1 was only 13 minutes at 37°, it was difficult to take more than 6 samples within this period and still make the differences in time fully significant. Preliminary experiments had shown that very rapid changes in the number of intracellular phage particles occurred which made it desirable that as many samples as possible be taken. The temperature was therefore reduced to 23°, where the length of the latent period varied between 80 and 100 minutes. Samples taken every 6 minutes at that temperature thus corresponded to samples removed at the rate of one a minute at 37°. The experiments were carried out in the following manner.

A rapidly growing culture of bacteria in SM containing 0·001 M Mg was infected with phage T1 at a ratio of 1 phage particle to 50 bacteria, following the method

described above. Immediately following re-suspension the total phage present was counted in order to determine the number of infected bacteria. The infected culture was then incubated at 23° and aliquots withdrawn at certain intervals, cooled and stored at 0°. At the same time another part of the infected culture was left undisturbed and the total phage present estimated at intervals. When all samples had been collected they were shaken for 20 minutes by the apparatus described above and phage estimations carried out at the end of this treatment. The results are shown in Fig. 1.

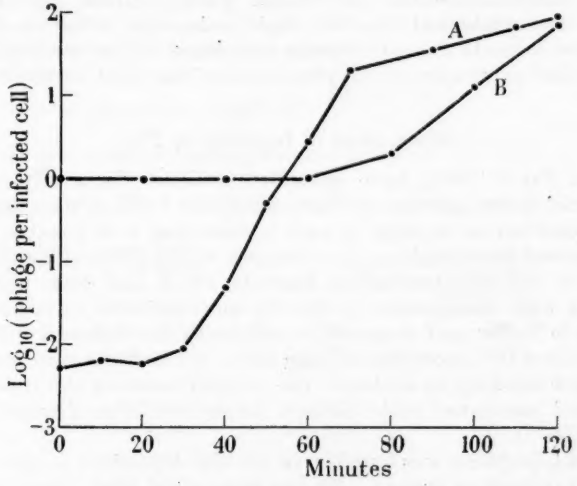

FIG. 1.—Multiplication of phage T1.

The experimental procedure is described in the text. Samples were withdrawn from an infected culture of bacteria and the total number of phages present assayed before and after disruption of the bacteria by the mechanical shaker.

Curve A: Average number of phages liberated per infected bacterium by artificial disruption.
Curve B: Average number of phages liberated per infected bacterium by natural lysis.

It is seen that over 99 per cent of the infecting phages immediately become non-demonstrable. According to current theories this is due to the fact that the infecting phage is broken down in the invaded cell (Rountree, 1951). A small percentage of phage, however, remained demonstrable. This may have been due to the fact that not all infected bacteria were disrupted, or that some bacteria were slower in reacting to the infecting phage particle than others.

Following this initial breakdown there was a short time during which the number of phage particles present remained constant. After about one-third of the latent period had elapsed phage began to multiply, and the number of phage particles demonstrable increased rapidly until 70 minutes and somewhat more slowly until 100 minutes after infection. Lysis of the undisturbed culture began 80 minutes after infection, and continued for some time until the number of phage particles demonstrable before and after shaking was the same. The burst size was not very high, as a temperature of 23° was below the optimum for *Bact.*

coli. This experiment was repeated using multiply-infected bacteria, with substantially the same result.

It was noted in experiments where the infecting multiplicity was close to one that infected bacteria soon after infection became viscid. This became more and more pronounced until just before lysis the bacteria could scarcely be re-suspended after centrifugation, but formed a single glutinous pellet. It was found that the bacteria could be restored to their normal state by treating them with the enzyme desoxyribonuclease, prepared by the method of McCarty (1946). A substance was dissolved off the cells having a pronounced absorption maximum at 270 mμ. Ribonuclease had no effect. Murray, Gillen and Heagy (1950) have shown in cytological studies that soon after infection UV-absorbing material moves towards the cytoplasmic membrane of the bacterial cell. This is probably another expression of the phenomenon described here.

First Steps in Infection by T1.

Garen and Puck (1951) have presented evidence for a rôle of mono- and di-valent metals in the infection of *Bact. coli* by T1. If, as they maintain, phage can remain adsorbed to bacteria in such a state that it is possible to elute it, it might be expected that crushing such bacteria would liberate the phage.

In order to test this possibility, bacteria which had been washed twice in distilled water were re-suspended in known concentrations of sodium and potassium phosphate buffer and magnesium sulphate, and infected with T1. After allowing 8 minutes for adsorption to take place, the bacteria were centrifuged and disrupted in the mechanical shaker. The phage present in the supernatant after adsorption, and associated with bacteria before and after disruption, was then estimated (Table I).

The rate of adsorption was found to be greatly dependent on the concentration of sodium and potassium present. It was most rapid when these metals were at a total concentration of 0·1 M and slowest at 0·0005 M. It was not affected by the presence of divalent metals at the higher concentrations, but at 0·0005 M sodium and potassium it was increased significantly by 0·001 M magnesium.

When however the number of " active " adsorptions was considered, the picture was quite different. An " active " adsorption was defined as one in which a phage particle was adsorbed on a bacterium in such a way that when plated out and incubated a plaque resulted. When such an infected bacterium was disrupted, however, it was found that there were two types of " active " adsorptions : (*a*) those in which the phage particle had been adsorbed on the bacterium but had not yet " penetrated " ; such a phage particle was thrown free by the shaking treatment and was demonstrable as a plaque ; (*b*) those in which the phage particle had already been broken down in the infected cell and where phage growth had commenced. This type of actively adsorbed phage particle did not form a plaque following the shaking treatment. An " inactive " adsorption was defined as one in which the phage particle had broken down in the bacterium, but for some reason phage multiplication had not occurred and no plaque resulted after plating.

Very few of the adsorptions in 0·1 M buffer were " active." Ninety per cent of the phage appeared to be broken down in the bacteria, but owing to the absence of energy sources no phage multiplication took place and most of the bacteria

TABLE I.—*T1* : *Effect of Mono- and Divalent Metals on Adsorption.*

Bacteria at a concentration of 4×10^8/ml. were washed twice in distilled water and re-suspended at the original concentration in sodium and potassium phosphate buffer (concentration given in column 1). The amount of magnesium or calcium salt added is stated in column 2. An accurately measured amount of phage (a) was then added to give a ratio of phage to cells of 1 : 4. After 8 min. incubation at 37° to allow adsorption to proceed, the infected bacteria were centrifuged and the supernatant assayed for phage (s). The bacteria were then washed once and the phage associated with them counted (r) after re-suspension in the same medium in which infection had taken place. They were then crushed in the mechanical shaker and assayed again (t). All operations were carried out in the presence of 0·05 per cent gelatin

Calculations :

Per cent adsorption (column 3) : $\frac{100\ (a - s)}{a}$.

Per cent " active " adsorptions (column 4) : $\frac{100\ r}{a}$.

Per cent " active " adsorptions stable to crushing (column 5) : $\frac{100\ t}{r}$.

Concentration of monovalent metals (Na + K).	Concentration of divalent metals (Ca or Mg).	Per cent adsorption.	Per cent " active " adsorption.	Per cent of " active " adsorptions stable to crushing.
0·0005 M	0·001 M Mg	45	30	60–80
,,	None	25	20	40–60
0·01 M	0·0016 M Mg	75	50	40
,,	0·002 M Ca	75	60	40
,,	None	75	30	40
0·1 M	0·0016 M Mg	95	10	0
,,	0·0005 M Ca	95	10	10
,,	None	95	10	10
Full synthetic medium	0·0016 M Mg	95	100	0
	None	95	100	0

permanently lost the ability to support phage growth. Of those infected bacteria that did retain their ability to form a plaque, about 10 per cent of the number initially infected, from 90 to 100 per cent did so by retaining the ability to support phage growth in the absence of an energy source until they were plated out on peptone agar.

In 0·01 M sodium and potassium phosphate the ratio of " active " to total adsorptions was higher, and increased by the presence of magnesium and calcium. A considerable proportion of adsorbed phages had not reacted with the bacteria within 8 minutes after infection and were thrown free by the shaking treatment.

In 0·0005 M buffer even more phage had not been broken down, and therefore survived the shaking treatment.

It should be emphasised that it was not possible to distinguish between phages which were broken down in the infected cell so that the latter could no longer form a plaque, and those that were inactivated by linkage with a phage receptor. There was the possibility that shaking broke the receptor-bacterium bond rather than that between phage and receptor, and that such a phage-receptor complex

was non-infective. As, however, there is considerable evidence that phage breaks down on infecting actively metabolising bacteria, it is likely that phage behaves similarly when adsorbed to resting cells. It might be postulated that in the absence of an energy source no synthesis of phage components can occur and that it does not recommence on the addition of metabolisable substrate. This argument is supported by the finding that the more care was taken to exhaust cells of reserve substrates, the greater was the percentage of "inactive" adsorptions.

Intracellular Growth of T2r+.

When intracellular growth of phage T2r+ was examined under the conditions outlined above, results similar to those obtained with T1 were recorded. Here, however, growth of phage in lysis-inhibited bacteria could also be studied (Doerman, 1948*b*). Bacteria growing in SM were infected at high and low multi-

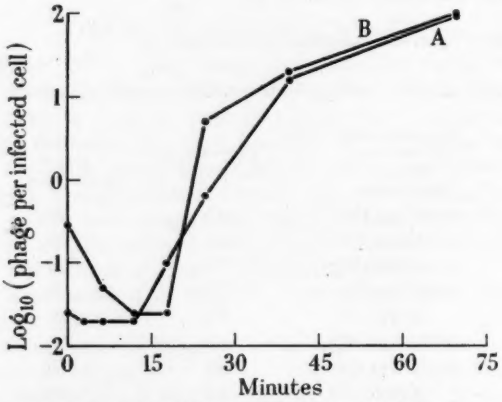

FIG. 2.—Intracellular multiplication of phage T2.

Two cultures of bacteria were infected with T2 at multiplicities of twenty and one-tenth. At intervals samples were withdrawn and the bacteria disrupted.

Curve A: Average number of phage particles liberated per singly-infected bacterium.
Curve B: Average number of phage particles liberated per multiply-infected bacterium.

plicities (twenty and one-tenth respectively) and the course of intracellular multiplication followed in both cases (Fig. 2).

It is seen that the two curves describing phage growth almost coincide except in the first few minutes. While in the case of single infection all adsorbed phages were very quickly broken down in the bacteria, in the case of multiply-infected bacteria this process took much longer. As the bacteria had been washed, the initial decrease in the number of active phages in the suspension of multiply-infected bacteria could not be due to adsorption to already infected bacteria, but was a reflection of the fact that several phages were broken down in each bacterium. Phage growth, however, proceeded at approximately equal rates in both singly- and multiply-infected cells. Doerman (1948*a*) found that in the presence of 5-methyl tryptophan or cyanide phage multiplication in multiply-infected cells started slightly ahead of that in singly-infected cells, but that the rate of phage formation was the same.

Extent of Phage Multiplication in the Absence of an Energy Source.

If infected bacteria were re-suspended in 0·06 M phosphate buffer instead of in synthetic medium, and intracellular phage growth then followed, it was found possible to demonstrate a slight amount of phage multiplication, but the newly formed phage was never liberated and disappeared again after some time (Fig. 3).

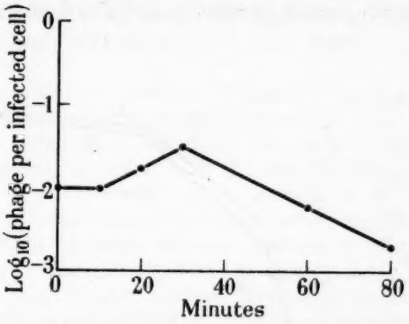

FIG. 3.—Intracellular multiplication of phage T2 in bacteria suspended in phosphate buffer.

Rapidly growing bacteria were infected, centrifuged and re-suspended in 0·06 M phosphate buffer. At various intervals samples were disrupted in the mechanical shaker and the average number of phage particles liberated per infected bacterium determined.

The Possible Existence of Aggregates of Phage.

The possibility was tested that phage multiplies in definite areas within the cell. Disruption of the bacteria might then conceivably release aggregates of phage with the individual members adhering to each other, with the result that the whole group would assay as one phage. Accordingly, bacteria which had been disrupted were incubated for 1 hour at 37° so that complexes might disperse. Phage was assayed before and after this treatment. A crystal of thymol was added to prevent the lysis of any uncrushed bacteria which could have distorted the result. As Fig. 4 shows, however, the titre after incubation was the same as before.

First Steps in Infection by T2.

The experimental technique described for T1 was followed. The results are summarised in Table II. In marked contrast to T1, it was found that in the

TABLE II.—*T2*: *Effect of Mono- and Divalent Metals on Adsorption.*

Conditions as described in Table I except that the adsorption time was 12 min.

Concentration of monovalent metals (Na + K).	Concentration of divalent metals (Mg).	Per cent adsorption.	Per cent "active" adsorption.	Per cent "active" adsorptions stable to crushing.
0·01 M	None	25	100	25–40
"	0·001 M	25	100	25–40
0·1 M	None	100	100	1
"	0·001 M	100	100	1

presence of both 0·1 and 0·01 M sodium and potassium phosphate 100 per cent of all adsorptions were "active." At the higher concentration almost all infecting phage was broken down in the bacteria, but at the lower concentration a considerable proportion of the phages had not reacted with the bacterial cells in 12 minutes, and therefore survived the shaking treatment. The addition of divalent metals had no effect. The rate of adsorption itself was related only to the concentration of monovalent metals present and did not change on the addition of divalent metals.

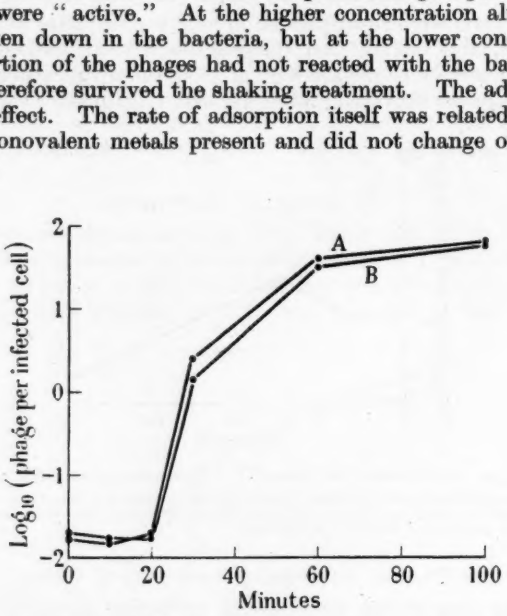

FIG. 4.—Attempted demonstration of the liberation of aggregates of phage by disruption of infected bacteria.

Phage was estimated before and after one hour's incubation at 37° of suspensions of infected bacteria disrupted by the mechanical shaker.

Curve A: Phage count per infected bacterium before incubation.
Curve B: Phage count per infected bacterium after incubation.

DISCUSSION.

In carrying out any research project the aim of which is to study virus reproduction, the technique of disrupting the host cell is likely to prove an extremely important one.

The technique presented here ensures that the bacteria are "fixed" *in statu quo* within a few seconds of taking a sample. By storing these samples at 0° one can be reasonably certain that all reactions are arrested until all samples have been collected, and they can then all be subjected to the disrupting treatment at the same time. The means used to disrupt the bacteria are purely mechanical and leave their contents undamaged for further studies.

The results presented here confirm those of Doerman (1948*a*, 1951) in that the infecting phage is broken down or in some way rendered non-infective. The rate at which this occurs appears to be influenced by the ionic concentration of the medium. At concentrations employed in the normal culture media, i.e., of the order of 0·1 M, this breakdown is an extremely rapid process, and it was possible to detect a gradual decrease in the number of intact adsorbed phage particles only if bacteria multiply infected with phage T2r$^+$ were employed. As the concen-

tration of electrolytes was lowered, the proportion of adsorbed phages "reversibly" attached to the bacteria increased.

The breakdown of phage occurred both in the presence and absence of energy sources. In the absence of energy sources immediate extensive phage growth is not possible, however, and can only commence when the infected bacterium comes into contact with the plating medium. In that interval between the breakdown of the invading particle and transfer to the medium containing energy sources T1 loses all ability to multiply, whereas T2 then starts to grow in the normal manner.

The reason for this difference between T1 and T2 is not clear, but there are other indications that T2 is a more powerful infective agent for *Bact. coli* strain B than T1. One may recall that Delbrück and Luria (1942) had found that if bacteria were simultaneously infected with T1 and T2 (which they called phages α and γ respectively) only T2 would grow; growth of T1 was only possible if infection with it preceded infection with T2 by several minutes.

The first demonstrable increases in the number of phages within infected bacteria occurred towards the middle of the latent period. The rate of increase was logarithmic rather than linear, but the technique used was not sufficiently refined to settle this point beyond doubt. The rate of phage multiplication in cells infected multiply was very similar to that in singly infected cells.

Towards the end of the latent period the rate of phage multiplication gradually declined until lysis occurred.

SUMMARY.

A method of analysing infected bacteria for intracellular phage is presented, which involves arresting phage multiplication by rapid cooling to 0°, and disruption of the bacteria in a mechanical shaker.

This technique has been used in investigating two problems:

(*a*) the intracellular growth of phages T1 and T2, using both singly- and multiply-infected bacteria;

(*b*) the nature of the adsorption of these two phages on bacteria suspended in solutions containing mono- and divalent metal ions but no source of energy.

I am deeply grateful to Sir Paul Fildes, F.R.S., for his advice and guidance during this investigation. I am also greatly indebted to Dr. D. D. Woods, F.R.S., Reader in Microbiology, University of Oxford, for placing at my disposal a shaker used during part of this work.

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THE EFFECT OF PHAGE INFECTION ON THE METABOLIC ACTIVITY OF THE HOST CELL.

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ONE of the problems connected with the multiplication of phage which has as yet received scant attention is that of the metabolic changes caused in the bacterium by the presence of phage inside it. Cohen (1948) has shown that infected bacteria do not synthesise ribonucleic acid but synthesise desoxyribonucleic acid at an increased rate. The syntheses of uracil and ribose are thereby suppressed, thymine and desoxyribose syntheses accelerated.

Luria and Human (1950) and Murray, Gillen and Heagy (1950) have made cytological studies of phage-infected bacteria and found that major changes in the distribution of cytoplasmic components take place. It might be expected therefore that various enzyme systems which depend on their spatial interrelationships for their maximum efficiency would be affected by infection. It has also been suggested (Luria and Human, 1950) that on infection the phages' genes replace bacterial genes as the directive agents for the specificity of proteins formed after infection. It is therefore not surprising that certain proteins, for instance adaptive enzymes, are no longer formed in the infected bacteria (Monod and Wollman, 1947).

Cohen and Anderson (1946) have followed the oxygen uptake of infected cells in the presence of lactate and found it to be but slightly below that of normal cells. The R.Q. following infection with T2 and T4 remained the same. Pardee (1951) compared the adenosine triphosphatase activity of normal and infected bacteria and found it to be increased in the latter. He offered several explanations for this.

In this paper some biochemical studies on *Bacterium coli* infected with phages T1 and T2 are reported.

METHODS.

The origin of strain B of *Bact. coli*, of the phages T1 and T2, the composition of the medium, and the method of preparing stock phage suspensions have been reported (Joklik, 1952). High titre (about 10^{12}/ml.) phage preparations were obtained by concentrating large volumes of lysates under reduced pressure, dialysing, and removing bacterial debris and denatured protein by centrifugation. Manometric studies were carried out with conventional Warburg equipment at 37°. The total volume per vessel was always 3·0 ml. Phosphate estimations were done by the method of Berenblum and Chain (1938); acid-labile phosphate was hydrolysed with N HCl for 7 minutes at 100° in sealed tubes.

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RESULTS.

The Effect of Infection on Certain Enzyme Systems in Growing Bacteria.

The first experiments on the effect of infection on the metabolic activities of bacteria were carried out with growing cultures. Small aliquots of such cultures were centrifuged, re-suspended in synthetic medium lacking an energy source and transferred to Warburg vessels. After equilibration at 37°, substrate (glucose or lactate) was added and the oxygen uptakes of the non-infected bacteria measured. Phage was then added to give a multiplicity of 5 and the rates of oxygen uptake followed as phage multiplication proceeded. Fig. 1 shows some

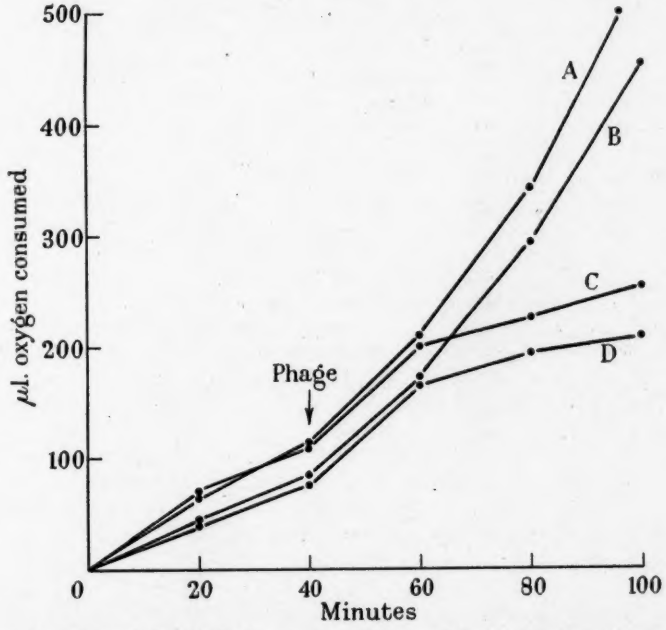

FIG. 1.—Effect of phage infection on the oxygen uptake with glucose and lactate.

(Each vessel (with two side-arms) contained 2·5 ml. bacteria (at 4 × 10^8/ml.) in ammonia-phosphate medium pH 7·4; glucose or lactate, 0·2 ml. 0·5 M; stock phage T1 (2 × 10^{10}/ml.), 0·3 ml.; and 0·1 ml. 10 N sodium hydroxide in the centre well.)

Curve A: Glucose, no phage.
Curve B: Lactate, no phage.
Curve C: Glucose, phage added at 40 minutes.
Curve D: Lactate, phage added at 40 minutes.

typical curves. The rate of oxygen uptake gradually ceased increasing as bacterial multiplication was inhibited by infection, and then decreased as the bacteria lysed.

Similar results were obtained when experiments were carried out under anaerobic conditions, when phage multiplication was only slightly slower than under aerobic conditions. Fig. 2 illustrates the CO_2 production from glucose.

The Relative Activities of Some Enzymes in Bacteria at Various Stages of the Latent Period.

In order to determine whether the decreases in enzymic activities noted above were due to lysis of the infected cells or occurred while they were still intact, the activities of a number of enzymes were estimated in bacteria which had been

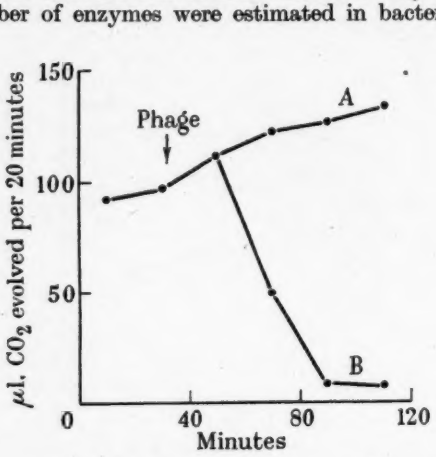

FIG. 2.—CO_2 production with glucose as substrate.

(Each vessel contained 3·0 ml. synthetic medium with phosphate replaced by bicarbonate. Bacteria 4×10^8/ml. with and without phage T1 10^9/ml. Atmosphere: 5 per cent CO_2 in nitrogen.)

Curve A: no phage added.
Curve B: phage added after 30 min.

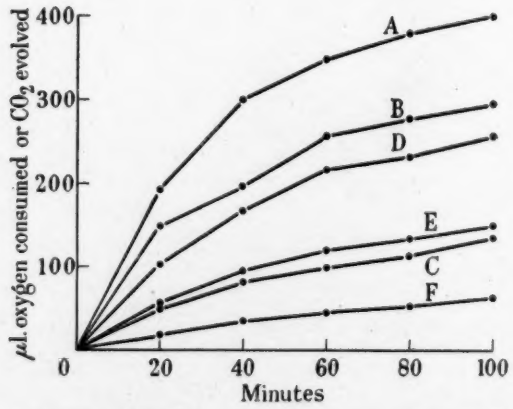

FIG. 3.—Oxidation of glucose and anaerobic glycolysis of infected bacteria.

(Warburg vessels contained cells infected with T1 for different lengths of time at a concentration of 10^8/ml. in synthetic medium for oxygen uptakes, and in synthetic medium with phosphate replaced with bicarbonate for the glycolysis experiments where the atmosphere was 5 per cent CO_2 in nitrogen.)

Curves A, B and C: Oxygen uptakes by bacteria infected for 8, 16 and 24 min. respectively.
Curves D, E and F: CO_2 evolutions by bacteria infected for 8, 16 and 24 min. respectively.

infected for various periods. Experiments were carried out in the following manner :

A culture of bacteria at 4×10^8 cells/ml. was infected with T1 at a multiplicity of 4. At 8, 16 and 24 minutes samples were removed, chilled to 2° and centrifuged. One half of each sample (A) was re-suspended at 2·5 times the original concentration in synthetic glucose medium, and the other half (B) in similar medium in which the concentration of phosphate had been greatly reduced and replaced with bicarbonate. The bacteria were transferred to chilled Warburg vessels so that no reaction could take place while the vessels were fixed to the manometers and while the manometers of series B were flushed with 5 per cent CO_2 in nitrogen. They were then incubated at 37° and the oxygen uptakes of series A and the CO_2 evolutions of series B measured (Fig. 3).

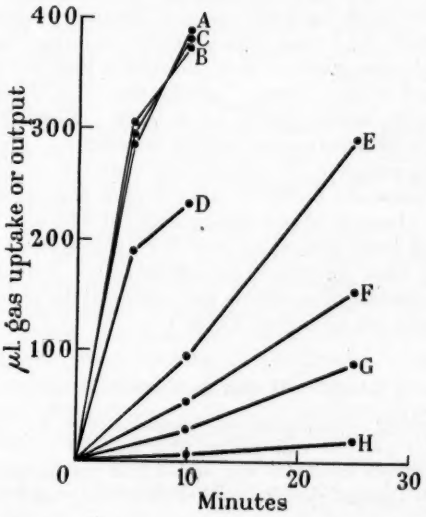

FIG. 4.—Catalase and hydrogenase activity of bacteria infected for different lengths of time.

(Vessels contained infected cells at 2×10^8/ml. Substrate for catalase : hydrogen peroxide, 3 per cent, 0·1 ml. : for hydrogenase, methylene blue, 1 per cent, 0·5 ml. in an atmosphere of hydrogen.)

Curves A, B, C and D : Oxygen evolution due to catalase by bacteria infected for 0, 8, 16 and 24 min. respectively.

Curves E, F, G and H :: Hydrogen uptakes due to hydrogenase by bacteria infected for 0, 8, 16 and 24 min. respectively.

Both oxidation of glucose and anaerobic glycolysis became progressively weaker the longer the bacteria had been in contact with phage. It should be mentioned that the reason that it was possible to obtain bacteria infected for 24 minutes and not yet lysed was that the original culture had not been growing rapidly as it had been incubated in a non-aerated vessel. The bulk of a vigorously growing aerated culture of bacteria normally lyses about 18 minutes after the addition of excess phage.

In a similar experiment the hydrogenase and catalase activities of bacteria infected for 8, 16 and 24 minutes were compared with those of non-infected bacteria (Fig. 4). The hydrogenase activity decreased during the latent period

as had been found for glucose oxidation and anaerobic glycolysis, but the activity of catalase remained much more constant. Infection thus caused certain changes in the bacterial cell which affected some enzymes more than others.

The Effect of Infection on Enzyme Activities in Resting Cells : Preliminary Experiments.

When working with infected bacteria which were about to lyse, it was always difficult to decide to what extent the decrease in activity of an enzyme system was due to the enormous dilution resulting from lysis. An attempt was therefore made to eliminate this difficulty by using cells in which the effect of infection could be studied without the complications caused by subsequent phage multiplication and lysis.

It had been noted that bacteria usually became glutinous some time after infection (Joklik, 1952), and that this occurred whether the bacteria were suspended in synthetic glucose medium or in phosphate buffer. Far-reaching changes were evidently caused by the mere " penetration " of a phage particle, even if no phage multiplication subsequently took place. Resting bacteria infected in phosphate buffer were therefore used for the succeeding experiments.

In the main compartment of a Warburg vessel was placed a suspension of bacteria in 0·06 M phosphate buffer of pH 7·6 at a concentration of 3 to 6 $\times$ 10^9 cells/ml. and enough phage to give a multiplicity of 3 to 4. In the controls phage was added which had been heated at 100° for 10 minutes ; this was completely inactive. The side arm received the substrate. Following incubation for various times, the substrate was added and the activity of the enzyme concerned measured. The results are shown in Table I.

TABLE I.—*Effect of Length of Infection Period on Certain Enzyme Systems.*

(Each Warburg cup contained bacteria (2·0 ml. 5 $\times$ 10^9/ml.) in phosphate buffer pH 7·6 and phage (0·5 ml. 1·5 $\times$ 10^{11}/ml.) mixed at time 0 ; at intervals substrate was added and the oxygen uptake over a definite, indicated period determined. Substrates : all 0·05 M.)

Length of infection period (minutes).	Reaction tested.	Gas uptake (µl.)* phage +	Gas uptake (µl.)* phage −
0	Glucose, O_2 uptake	170	161 per 10 minutes
30	" "	148	152 " 10 "
90	" "	162	148 " 10 "
0	Pyruvate, O_2 uptake	53	63 " 10 "
60	" "	24	58 " 10 "
15	Nitrate, H_2 uptake	133	105 " 15 "
120	" "	146	123 " 15 "
15	Lactate, O_2 uptake	36	38 " 15 "
135	" "	47	44 " 15 "
15	Glycine, O_2 uptake	47	108 " 60 "
60	" "	29	84 " 60 "

* Due correction for the endogenous gas uptake or output has been made in this and the following tables.

The oxidations of glucose and lactate and the reduction of nitrate with molecular hydrogen were not affected by infection, but the rates of oxidation of pyruvate and glycine were greatly decreased, the latter after only 15 minutes' contact between phage and bacteria.

Although these were concentrated suspensions of resting bacteria, some lysis occurred after the addition of the substrate, i.e., of an energy source, and at first made it doubtful how much of a decreased enzymic activity was due to a decreased number of cells present. Thus, in one experiment, where the concentration of bacteria initially was $6{\cdot}0 \times 10^9$/ml., it had decreased to $5{\cdot}5 \times 10^9$/ml. after 90 minutes' incubation with excess T1 in phosphate buffer. Glucose was then added to one sample, and after 30 minutes the infected bacteria had decreased to $5{\cdot}0 \times 10^9$/ml., whereas the non-infected bacteria were still at $6{\cdot}0 \times 10^9$/ml. The oxygen uptakes during those 30 minutes were 231μl. for the non-infected bacteria, and 255μl. for the infected. To another sample of the culture alanine was added. After 60 minutes' incubation time the infected culture had decreased to $4{\cdot}5 \times 10^9$/ml., the blank to $5{\cdot}8 \times 10^9$/ml. The oxygen uptakes were 145μl. for the non-infected, and 60μl. for the infected bacteria.

This small amount of lysis could be decreased by aerating the bacteria in phosphate buffer before the start of the experiment. Exhaustive washing of the cells also eliminated this small amount of lysis, but caused a relatively big decrease in the activity of the enzymes tested.

It was concluded from these results that although a certain small amount of lysis sometimes occurred, the far more important cause of the decreased activity of certain enzyme systems was infection of the cells. This was confirmed by the fact that the metabolism of a strain of resistant bacteria was quite unaffected by T1. This strain was obtained by plating out so many phages on a a plate that the plaques were confluent. Continued incubation enabled the resistant bacteria to form colonies, and one of these was picked off and subcultured several times. The effect of infection on the parent susceptible strain and the resistant strain was then compared with regard to the oxidation of alanine, acetate and reserve substrate (Table II). Care was taken to have the cells of both types at the same concentration.

TABLE II.—*Effect of Infection on Certain Oxidations by Susceptible and Resistant Bacteria*

(Each Warburg vessel contained bacteria (2·0 ml. at a concentration of 5×10^9/ml.) in phosphate buffer pH 7·6 and phage T1 (0·5 ml. at a concentration of $2{\cdot}15 \times 10^{11}$/ml.). Side arms contained the substrates: alanine, 0·5 ml. 0·2 M; acetate, 0·5 ml. 0·4 M; endogenous, no substrate added. Centre wells contained potassium hydroxide, 0·2 ml. of 10 M. Incubation time before addition of substrate, 60 min.)

	Type of cells.											
	Susceptible.						Resistant.					
Substrate	alanine		acetate		endogenous		alanine		acetate		endogenous	
Phage present	+	−	+	−	+	−	+	−	+	−	+	−
	Oxygen uptake in µl.											
0–15 min.	21	18	3	2	49	71	48	35	9	0	112	116
0–45 „	63	81	12	19	135	189	197	188	33	22	279	284
0–75 „	115	161	19	34	198	278	430	412	159	137	340	350

The oxidations of both alanine and acetate were adaptive in the strain of *Bact. coli* used here; that is, the rate of oxidation of these two substrates progressively increased in the absence of cell multiplication. This adaptation was completely inhibited by infection. Thus the rate of oxidation of acetate and alanine of susceptible infected bacteria remained constant at the initial level, whereas that of the susceptible bacteria to which heat-inactivated phage had been added and of resistant bacteria in contact with either active or heat-inactivated phage increased. This adaptation was made possible in the absence of an energy source and of an easily utilisable carbon source by the reserve substrates present in the bacteria. That such were present was indicated by the high endogenous rate of oxygen uptake, i.e., the oxygen uptake in the absence of an added substrate. This was also partially inhibited by infection, but at other times it was not affected. The particular strain of resistant bacteria used here showed a much higher rate of adaptation to acetate and alanine oxidation than the parent susceptible strain.

Survey of Enzyme Systems.

When it had thus been shown that any decrease in activity of any enzyme system in phage-infected cells was not due to lysis, but to the presence of active phage, and that there was no substance in an active phage suspension which would affect the activity of enzyme systems in resistant bacteria, a broad survey was made of the effect of phage infection on a number of enzymes and enzyme systems. The general procedure was to use a cell suspension of about 5×10^9

TABLE III.—*Effect of Phage Infection on Enzyme Activities.*

Reactions tested.	Results and comments.
Oxygen uptake on glucose	No effect.
,, ,, lactate	,,
,, ,, formate	,,
,, ,, fumarate	,,
,, ,, malate	,,
Hydrogen uptage on nitrate	,,
Oxygen uptake on pyruvate	50 per cent inhibition.
,, ,, succinate	60–80 per cent inhibition.
,, ,, glycine	50 per cent inhibition.
,, ,, endogenous substrates	Generally not inhibited.
,, ,, alanine	Inhibited 30–50 per cent. When adaptation occurred completely inhibited.
,, ,, acetate	Adaptation to acetate oxidation inhibited. Oxygen uptake without adaptation usually negligible
Anaerobic CO_2 production from glucose	40 per cent inhibition
Anaerobic CO_2 production from pyruvate	,, ,,

bacteria per ml., phage T1 to give a multiplicity of 4 — 5 to 1, and to incubate them together in 0·06 M phosphate buffer (with 0·001 M Mg) for 1 hour in Warburg flasks. Then substrate was added and manometer readings taken for 30 to 60 minutes. Table III summarises the results.

While the oxidation of certain substrates was not affected by infection, that of others was greatly inhibited. Into the former group fell the oxidations of glucose, lactate, formate, fumarate and malate, and generally the oxidation of reserve substrate was only slightly inhibited. To the latter category belonged pyruvate, succinate, glycine, and also alanine and acetate oxidation. The oxidation of these two substrates, as mentioned above, was adaptive and the adaptation was inhibited by infection.

Anaerobic CO_2 production from both glucose and pyruvate was inhibited in infected bacteria. The reduction of nitrate with hydrogen, on the other hand, was not. Judging from the distribution of enzyme systems in the two groups, (*a*) those inhibited by infection, and (*b*) those not affected, there seemed to be no way of predicting whether any particular reaction would be affected or not.

Although it had been shown that none of these results was attributable to lysis of the infected cells, it seemed desirable to eliminate the possibility of lysis altogether. This was achieved by infecting bacteria with phage T2r$^+$ at a multiplicity greater than one. This leads, as Doerman (1948) has shown, to the phenomenon of lysis inhibition, which is characterised by the fact that any cell infected by more than one phage particle does not lyse after the normal latent period, but only after a period of 3 to 6 hours. Table IV shows that phages T2r$^+$ and T1 inhibited the oxidation of pyruvate and succinate to a similar degree.

TABLE IV.—*Comparison between the Effects of T1 and T2 on the Metabolic Activities of Infected Bacteria.*

(Each Warburg vessel contained bacteria (2·0 ml. at a concentration of 5×10^9/ml.); phage (T1 or T2 at a concentration of $1{\cdot}5 \times 10^{11}$/ml.); and substrate, 0·5 ml. 0·4 M.)

Substrate.	Oxygen uptake in µl. per 30 min.		
Phage ⟶	T1	T2	None
Pyruvate	92	53	296
Succinate	12	23	60

TABLE V.—*Effect of T2 Infection on the Oxygen Uptakes of Acetate-grown Bacteria.*

(Contents of Warburg vessels as for Table IV. "+" infected bacteria, "—" non-infected bacteria.)

Substrate.	Oxygen uptake (µl.).			
	0–15 minutes.		15–30 minutes.	
	(—)	(+)	(—)	(+)
Glucose	231	222	261	220
Acetate	225	190	227	206
Succinate	156	168	142	150
Pyruvate	187	107	175	91

In all these experiments the bacteria were grown in synthetic glucose medium (Joklik, 1952). Cells grown in this medium rapidly oxidised glucose, pyruvate and lactate, but attacked acetate and succinate very slowly. However, when grown in either 1 per cent acetate + 0·02 per cent yeast extract, or in 1 per cent peptone, in both cases with vigorous aeration, the cells oxidised not only glucose and pyruvate but also acetate and succinate at a rapid rate. The oxidation of acetate and succinate by these bacteria was unaffected by infection with T2, but pyruvate oxidation was inhibited almost 50 per cent (Table V).

Synthetic Reactions.

All the reactions so far described are energy-yielding reactions. In order to determine the effect of infection on a synthetic reaction also, the synthesis of energy-rich phosphate bonds coupled to the oxidation of acetate and succinate by bacteria adapted to those two substrates was studied. This reaction has been studied in normal bacteria by Hersey and Ajl (1951). Five experiments were carried out altogether and are summarised in Table VI. A typical experiment is described in detail, and where conditions were altered this is indicated in the Table.

TABLE VI.—*Synthesis of Acid-labile (Energy-rich) Phosphate by Infected and Non-infected* Bact. coli.

(For experimental procedure see text.)

Substrate.	Added AA or ATP.	Phage.	Period of expt. (min.).	Oxygen uptake.	Acid-labile phosphate (μM). Initial.	Acid-labile phosphate (μM). Final.	Change. (μM phos.)
Acetate	ATP	+	60	300	6·5	4·1	−2·4
,,	,,	—	60	300	6·3	3·6	−3·3
Succinate	,,	+	60	96	12·6	8·7	−3·9
,,	,,	—	60	96	13·6	9·5	−4·1
,,	,,	+	120	1082	14·8	14·4	−0·4
,,	,,	—	120	1556	12·6	9·0	−3·6
,,	AA	+	60	88	..	..	+0·6
,,	,,	—	60	88	..	..	+1·2
,,	,,	+	60	Not recorded	3·5	4·4	+0·9
,,	,,	—	60	Ditto	3·2	8·3	+5·1

Bacteria were grown in peptone-acetate and were fully adapted to both acetate and succinate. In four experiments the bacteria from about 1 l. of medium were centrifuged towards the end of the log phase of growth (about 10^9/ml.), washed and re-suspended in phosphate buffer. One half was infected at a multiplicity of 5 with high titre dialysed T2 stock, and the other treated similarly with heat-inactivated T2. In one experiment the cells were infected in the peptone medium itself; the culture receiving the heat-inactivated phage was cooled to room temperature and the other, with active phage, incubated at 37°. After 90 minutes' infection the bacteria were centrifuged in all cases. Infected bacteria were always very glutinous. All following manipulations were carried out in the cold. The bacteria were ground with 200-mesh alumina

according to the method of McIlwain (1948), diluted with 0·013 M phosphate buffer and centrifuged. It was found that the longer the extract was centrifuged, the more inactive was the clear supernatant. The aim was therefore to include as much cell débris which had a high activity, without any whole cells, as possible. This was a matter of some difficulty, and led to some variations in the activity from one experiment to the next.

Reagents were then added to both extracts to give the following final concentrations: acetate, 0·05 M (one experiment only); ammonium succinate, 0·05 M; fluoride, 0·066 M; Mg, 0·001 M; a trace of phosphate acceptor (adenylic acid (AA)) in some experiments, and adenosine triphosphate (ATP) in others. These reaction mixtures were incubated in Warburg vessels for 1–2 hr., during which the rate of oxygen uptake was observed. Aliquots were removed at the beginning and end of the incubation period, and inorganic phosphate estimated both before and after hydrolysis of acid-labile phosphate bonds.

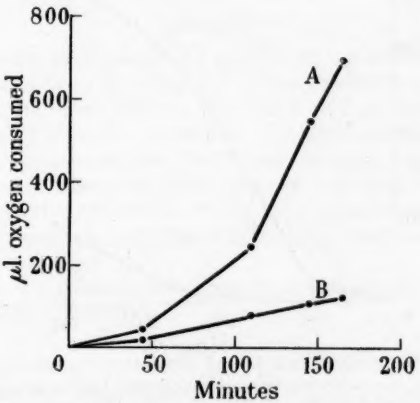

FIG. 5.—Adaptation to lactose oxidation by infected and non-infected bacteria.

(Log phase bacteria were used at a concentration of 10^9/ml. Medium: peptone, 0·5 per cent, lactose, 0·05 M.)
Curve A: Non-infected bacteria.
Curve B: Infected bacteria.

The results, shown in Table VI, indicate that when ATP was supplied initially there was a decrease in acid-labile phosphate which was greater in non-infected than in infected bacteria. When AA was supplied as phosphate acceptor acid-labile bonds were synthesised. This synthesis was greater in normal than in infected bacteria. The actual increases in acid-labile phosphate observed, however, were very small.

The Formation of Adaptive Enzymes.

It has been pointed out above that the ability of bacteria to adapt to acetate and alanine was completely inhibited by infection. Adaptation towards lactose oxidation was also inhibited in bacteria infected with phage T2 (Fig. 5). This had been discovered by Monod and Wollman (1947), who were working with a different bacterium-phage system.

An entirely different result was obtained with formic hydrogen lyase. Stephenson and Stickland (1933) have shown that this is an adaptive enzyme in *Bact. coli* and have worked out the conditions under which it is formed. Fig. 6 shows that although adaptation occurred with non-infected bacteria, infected bacteria adapted much more quickly.

DISCUSSION.

There is little doubt that far-reaching changes are caused in the bacterial cell by infection. Thus it has been found that the distribution of nuclear material changes in a characteristic way following infection (Luria and Human, 1950; Murray *et al.*, 1950), and that infected bacteria secrete a substance which renders them viscid and which can be removed with desoxyribonuclease (Joklik, 1952).

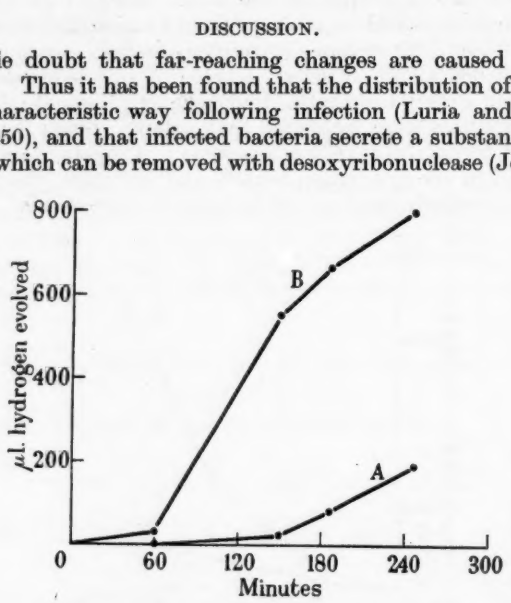

FIG. 6.—Adaptive formation of formic hydrogen lyase.
(Bacteria and medium as for Fig. 5; formate, 0·1 M. Atmosphere: Nitrogen. Centre wells contained 0·1 ml. 10 N sodium hydroxide.)
Curve A: Non-infected bacteria.
Curve B: Infected bacteria.

The activities of certain enzymes are also affected by infection. It is not clear exactly how the "penetration" of a phage particle could affect any particular enzyme. Only a limited number of enzyme systems have yet been studied before and after infection, and it is too early to decide whether any particular type of reaction is inhibited more than any other. So far however it appears that the effect of infection is non-specific and indirect. The disorganisation caused by infection, which has been discussed above, might, for instance, lead to the disruption of spatial interrelationships and so to decreased activities of certain enzyme systems. But it is also significant that the synthetic activity of the infected cell is restricted mainly to components of phage. That this is so is illustrated by the fact that infection prevented the synthesis of three out of four of the adaptive enzymes investigated here, and that even the activities of so-called "constitutive" enzymes, e.g., hydrogenase and catalase, did not increase after infection of growing bacteria, but remained constant, showing that the formation of these enzymes had been inhibited. Therefore,

as proteins within a cell are in a state of dynamic equilibrium, they would disappear from the cell. Unfortunately very little is known about the oxidation in *Bact. coli* of some of the substrates tested here, so that it is not possible to say whether disruption of spatial interrelationships or inhibition of their formation under conditions when their breakdown proceeded unchecked was the cause of their lowered activity, or whether the explanation has to be sought elsewhere.

The case of formic hydrogen lyase, which Woods (1936) has shown to be a reversible enzyme system, is very interesting, and it may well be that it has some special significance. Following infection desoxyribonucleic acid is synthesised to the exclusion of ribonucleic acid (Cohen, 1948), a change that involves the synthesis of thymine instead of uracil. These two pyrimidines differ only in that thymine has one more methyl group than uracil. Formate has recently been implicated in methylation reactions, and is perhaps involved in the interconversion of these two pyrimidines.

SUMMARY.

The effect of infection with phages T1 and T2 on the activity of certain enzyme systems of *Bact. coli* has been investigated. The oxidation of certain substrates, among them glucose, lactate, formate, fumarate and malate, was not affected, while that of others, pyruvate, succinate, acetate, alanine and glycine, was inhibited. The hydrogenase-nitrate reductase system was not affected, but anaerobic carbon dioxide liberation from glucose and pyruvate was inhibited.

The adaptation towards oxidation of acetate, alanine and lactose was completely inhibited by infection. In marked contrast, the formic hydrogen lyase system was greatly stimulated.

These findings are discussed in the light of the changes known to be induced in a bacterium by phage infection.

I would like to express my gratitude to Sir Paul Fildes, F.R.S., for his interest and advice throughout this investigation.

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SUPPRESSION OF THE TUBERCULIN REACTION IN RABBITS BY TOTAL BODY IRRADIATION WITH X RAYS.

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In those immune reactions in which antibodies are liberated into the blood stream, the effect of total body irradiation has been long known and widely studied (Benjamin and Sluka, 1908; Läwen, 1909, quoted by Colwell, 1935; Hektoen, 1915, 1918, 1920; Murphy and Sturm, 1925; Clemmesen, 1939; Clemmesen and Andersen, 1948; Craddock and Lawrence, 1948). In all cases the same type of response occurs: radiation near the time of introduction of the antigen prevents, weakens or delays the normal rise of antibody titre, while radiation a few days after the introduction of the antigen is without any such effect. That those immune reactions in which no circulating antibodies are demonstrable might also be similarly affected was first shown (if we except the equivocal work on transplanted tumours (Murphy, 1914)) by our own experiments on skin homotransplants in rabbits (Dempster, Lennox and Boag, 1950). It seemed worth while to extend these experiments to another better known and in some respects simpler reaction, tuberculin sensitivity, in which also no circulating antibody has been demonstrated, and whose analogies with the homotransplant reaction have been stressed by Burnet and Fenner (1949). Though some inconclusive work has been done on the effect of irradiation on the course of tuberculosis in experimental animals (Murphy and Ellis, 1914; Morton, 1916; and Kellert, 1918–19), we have been unable to find any published account of the behaviour of the tuberculin reaction under such circumstances.

From the known effect on other immune responses we predicted that irradiation in sufficiently high dose before or shortly after inoculation with tubercle bacilli would delay the onset of the tuberculin reaction, but that once positive the reaction would not be affected by irradiation. The experiments to be described were directed to the establishment of these two points and, when the second prediction was found to be false, to finding a cause for the discrepancy.

MATERIALS AND METHODS.

Animals.

We could only use rabbits as our experimental animal, since with the guinea-pig, the only other available animal in which tuberculin sensitivity can be demonstrated, the radiation dose levels required to produce an adequate response were usually lethal. Young adult male rabbits weighing 1·5–2·4 kg. were used throughout.

Infecting organism.

Two methods of infection were used (*a*) B.C.G. vaccine (State Serum Institute, Copenhagen) injected subcutaneously in a dose of 0·2 ml., and (*b*) human strain H 37 Rv, injected intravenously in a dose of 1 ml. of a 1:1000 dilution of an 18-day culture in Tween 80 albumin liquid medium. For the last group of experiments B.C.G.-infected rabbits (whose sensitivity had declined during an unavoidable delay of six months since inoculation) were reinfected with the human strain (see Table III).

Tuberculin reactions.

Mantoux-type tests were used throughout, 0·1 ml. of the tuberculin dilution being injected intradermally into the shaved skin of the back of the rabbits. For the earlier experiments with B.C.G. vaccine a commercial human-strain Old Tuberculin was used at a dilution of 1:100 (1 mg. O.T.); higher doses produced non-specific reactions. For the later experiments, P.P.D. (Ministry of Agriculture Veterinary Laboratory, Weybridge, human strain) was used. This could be employed at a strength of 0·2 mg./ml. (nearly equivalent to 1:10 O.T.) without producing non-specific reactions; this proved a considerable advantage since, possibly because most of the rabbits were older, the general levels of sensitivity were lower in the later groups.

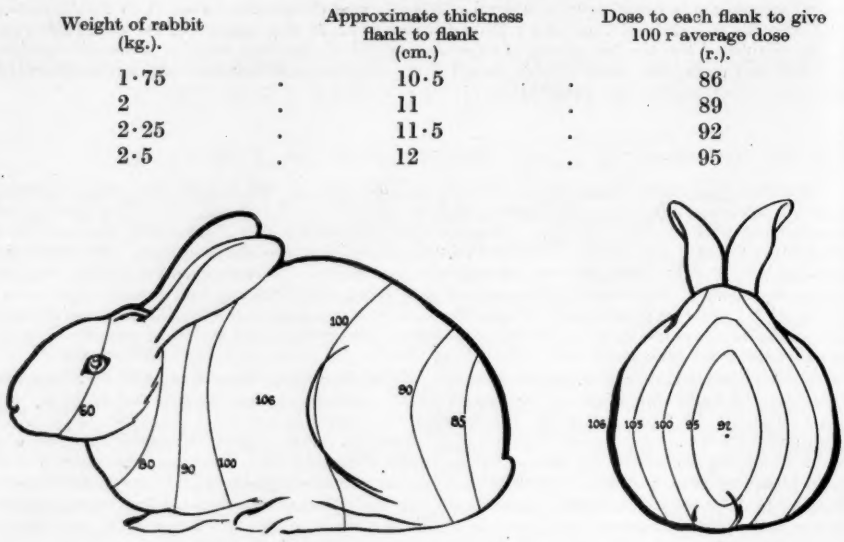
A positive reaction was recorded in terms of the diameter of the area of induration produced by the highest strength of tuberculin used, erythema being disregarded and readings being made at 40–48 hr. after the inoculation. A reaction has been recorded as negative if no palpable wheal was present 40 hr. after injection of the highest strength of tuberculin used. Inability to locate the lesion by palpation with the eyes shut may be accepted as good evidence of a negative reaction in a test in which complete objectivity is difficult to maintain. The three-level dose method of Long and Miles (1950) was used in many experiments, but, chiefly because it is unusual to find a rabbit in which 0·1 mg. O.T. produces a lesion well-defined enough to be measured, the results we have obtained do not appear to be suitable for mathematical treatment, and we have therefore recorded our results in terms of diameters at one dose level only. This appears to have been sufficient for our purposes. It may however be of interest to record the mean lesion diameters in eight non-irradiated rabbits in whom satisfactory responses were obtained at three levels. With 20 μg. P.P.D. (equivalent to 10 mg. O.T.) this was 15·7 mm.: with 2 μg. P.P.D. (1 mg. O.T.) 8·8 mm.; and with 0·2 μg. P.P.D. (0·1 mg. O.T.) 4·3 mm.

Technique of Irradiation and Dose Distribution.

The arrangements for the irradiation of the rabbits were identical with those described in the earlier paper. The X rays were generated at 190 kV. and filtered through 0·8 mm. Cu + 1 mm. Al, the half value layer of the radiation being 1·2 mm. of copper. The rabbit was confined in a wooden box with perspex sides and was irradiated first through one flank and then, for an equal time, through the other, the target-skin distance being 40 cm. This technique does not achieve a completely uniform radiation dose throughout the body of the rabbit, and as we have met some criticism based on the possible effect of irregular dosage, we have thought it worth while to make a careful exploration of the radiation intensity distribution in a rabbit carcass. Condenser ionization chambers about 1 $cm.^3$ in size, of the type generally used for depth dose measurements in radiotherapy were used. Several of these were inserted into celluloid tubes passing through the carcass which was irradiated in the usual way. The chambers thus recorded the dose at points inside the carcass. The results of this survey are represented in Fig. 1. The contours of equal radiation intensity are indicated on the flank and the variation inside the animal from flank to flank is shown on the rear view. The contours are labelled in per cent of the average dose, and it is this average dose which is quoted throughout the paper. Most of the body received within 10 per cent of the average, only a small part of the rump and the head and neck receiving less. The irradiation time required to deliver the desired average dose varied with the size of the animal in the manner shown in Table I, but the general distribution of dose illustrated in Fig. 1 was practically the same for all the rabbits used.

TABLE I.—*Effect of Weight and Thickness of Rabbit on X-ray Dosage under Standard Conditions.*

Weight of rabbit (kg.).	Approximate thickness flank to flank (cm.)	Dose to each flank to give 100 r average dose (r.).
1·75	10·5	86
2	11	89
2·25	11·5	92
2·5	12	95



(*a*) (*b*)

FIG. 1.—Contours of equal radiation dose on (*a*) the surface and (*b*) a central section of the rabbit, expressed in per cent of the average dose.

RESULTS.

Postponement of Appearance of Positive Tuberculin Reaction.

The observations on eight rabbits recorded in Table II show that the positive tuberculin reaction can be postponed by irradiation just before inoculation with

TABLE II.—*Postponement of Appearance of Positive Tuberculin Reaction by Irradiation.*

Rabbit.	Irradiation. Dose.	Irradiation. Day after injection.	Total dose.	Intradermal tuberculin.	Death.
TAD	230 r	1st, 15th, 36th and 50th	920 r	Negative until death	57th day
TAI	230 r	1st, 15th and 36th	690 r	,, ,,	49th day
TAL	160 r	1st, 15th and 29th	480 r	Positive from 60th day	Survived
TAM	160 r	,,	480 r	Negative* indefinitely	,,
TAN	160 r	,,	480 r	Negative until death	71st day
TAO	160 r	,,	480 r	Positive from 60th day	70th day
TAQ†	400 r	1st	400 r	Negative* indefinitely	Survived
TAR†	400 r	29th	400 r	Positive from 81st day	,,

All rabbits were given B.C.G. vaccine on 1st day. Fourteen of fifteen non-irradiated controls became positive within 36 days. Mantoux tests were done weekly, using 1:100 O.T.

* Persistently negative on weekly tests up to 109th day and at 3rd and 6th month.

† Other rabbits receiving these doses dying before the 35th day are not included.

B.C.G. vaccine, or in the succeeding few weeks. It will be seen that the irradiated rabbits were all negative up to the 60th day, while in controls all conversions occurred before the 35th day (the only exception among 15 control rabbits remained negative indefinitely). It would have been interesting to put these results on a more quantitative basis by further experiment; however, the suppression effect, next to be described, introduced so many possible complications in the interpretation of any but the grossest postponement effects that it was thought wiser to leave the matter, at least for the present, with this simple demonstration of the existence of the predicted postponement.

Suppression of Positive Tuberculin Reactions.

Rabbits which had become tuberculin-positive were irradiated on 19 occasions (Table III). Taking the results of intradermal injection of tuberculin on the

TABLE III.—*Suppression of Positive Tuberculin Reaction by Irradiation.*

Rabbit.	Infecting organism.	Tuberculin used.	Diameter (mm.) of reaction. Before irrad. (a)	Before irrad. (b)	After irrad. (c)	After irrad. (d)	Dose of X ray (r.)	Duration of suppression (approx.).
TAB	B.C.G.	O.T. (1 mg.)	15	18	–	0	600	4 weeks
TAC	,,	,,	12	30	0	0	400	6 ,,
TAG	,,	,,	18	12	6	6	230	—
TAT	,,	,,	20	12	0	0	200	10 days
TAV*	,,	,,	12	10	6	0	200	10 ,,
TAW	,,	,,	10	11	0	0	200	10 ,,
THAU	Human	P.P.D. (0·02 mg.)	19	17	12	12	200	—
THAX	,,	,,	17	15	4	6	200	—
THAY	,,	,,	16	16	13	0	200	14 days
THAG	B.C.G. and human	,,	6	6	0	0	200	14 ,,
THAH	,,	,,	5	6	0	0	200	14 ,,
THAI	,,	,,	15	11	0	6	200	—
THAJ	,,	,,	12	14	6	7	200	—
TAV*	,,	,,	13	13	6	0	200	21 days
THAA	,,	,,	22	24	15	6	200	—
THAD	,,	,,	7	6	5	0	200	14 days
THAE	,,	,,	17	17	6	0	200	14 ,,
THAF	,,	,,	17	17	12	7	200	—
TAK	,,	,,	12	14	9	8	200	—

Exact times for the tuberculin reactions recorded in columns *a–d* have not been given as they varied somewhat from rabbit to rabbit. Columns (*a*) and (*b*) represent the two last pre-irradiation tests, all within 14 days, usually on the 10th and 3rd days respectively before irradiation. Column (*c*) gives the smallest diameter of the lesion on the first day after the irradiation, and column (*d*) the level reached on the second day. Times given refer to the time of injection of tuberculin, and not of reading the result.

* Rabbit TAV was used twice with an interval of nearly a year, a booster inoculation of human strain bacilli having been given in the meanwhile. It shows a notable constancy of response.

second day after irradiation (column " d ") as standard, 11 of the rabbits showed complete suppression of palpable induration. One more (THAI) showed a transient suppression in the first 24 hr.; and every rabbit showed at least some decrease of lesion diameter. Even the least of these decreases, those for THAU and TAK, appear to be significant when compared with the differences between successive tests before irradiation (columns " a " and " b " of Table III). The least decrease, when expressed as a percentage of the mean of the pre-irradiation

tests, is more than three times the standard deviation of the percentage difference between the pairs of pre-irradiation tests.

After 200 r the suppression lasts for 10–20 days : with the two rabbits given higher doses the duration was considerably longer.

Timing of suppressive effect.

Tuberculin tests were done at various intervals after irradiation, and it soon became evident that suppression of the positive reaction was not abrupt (compare columns " c " and " d " in Table III). A fuller study of this was made in the three rabbits (Fig. 2). In each of these a series of intradermal tuberculin injections was done at intervals from two days before up to four after irradiation, and the lesion resulting from each of them was followed in detail until it disappeared. The gradual subsidence of the positive reaction is well seen in the successive graphs. In view of the obvious criticism that this might be due to desensitization produced by successive injections, similar serial reactions were done in the non-irradiated rabbits : maximum diameters at the end of such experiments tended to be a little lower than at the beginning but the effect, if any, was negligible.

Effect of local screening.

In four rabbits one end of the animal was screened from the radiation by a lead sheet. The reaction in the screened area was then compared with that in the standard site. Table IV shows that such local screening has little or no effect on the results ; in one animal only a negative reaction in the normal site was matched

TABLE IV.—*Comparison of Tuberculin Reactions in Screened and Unscreened Areas on Second Day after Irradiation.*

Rabbit.	Screened area.	Lesion diameter in screened area (mm.).	Lesion diameter on back (mm.).
THAD	Rump	0	0
THAH	,,	3	0
THAX	Head	6	7
TAV	,,	0	0

by a minimal reaction in the screened area. Measurement with an ionization chamber showed that the screened areas received less than 0·5 per cent of the dose given in the exposed areas. Incidentally, the lead sheet, when at the head end, screened the pituitary almost completely.

Effect of Irradiation on Course of Tuberculosis.

No macroscopically visible lesions were seen at autopsy in any of the B.C.G.-infected rabbits. Of the rabbits given human strain tubercle bacilli six months after B.C.G. and then irradiated, several have died with active tuberculous lesions, but as no control series is available, no conclusions can be drawn from these. The events in one group of five rabbits, infected with human strain only, seem, however, worth mention. Three of these were irradiated ; of these, one (THAY) died three weeks later with numerous tubercles ; two (THAU and THAX) died 2–3 months

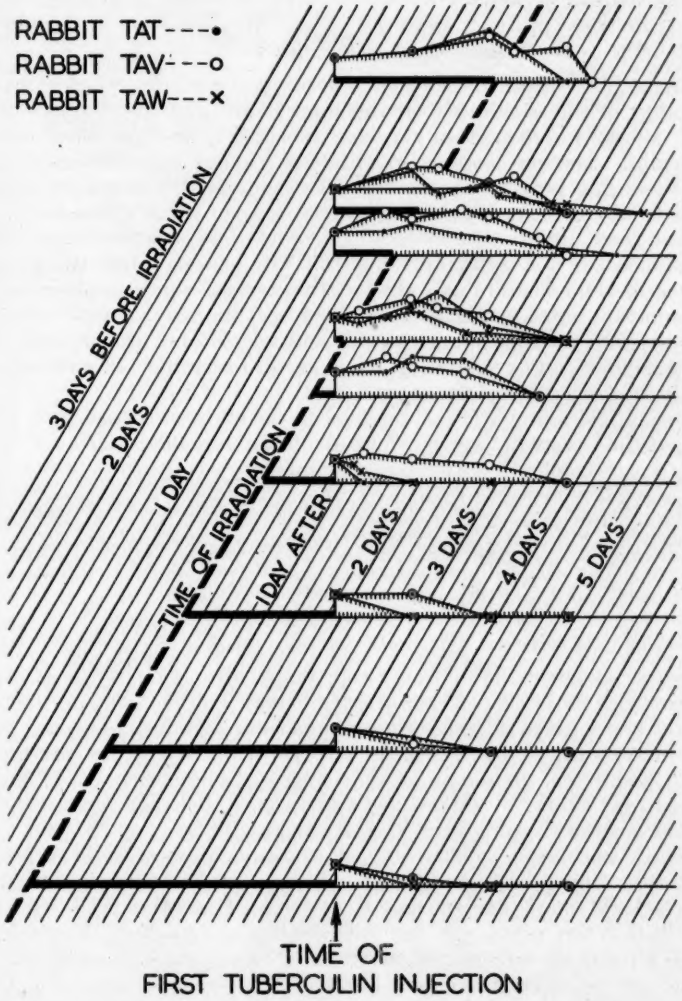


FIG. 2.—Time relationship of irradiation and the suppression of the tuberculin reaction in three rabbits. Each rabbit received a single dose of 200 r at zero time, and intradermal tuberculin (1/100 O.T.) at intervals before and after irradiation. Oblique axes have been used to facilitate comparison of the graphs showing the course of the lesions by bringing them vertically above each other. The graphs indicate the course of the diameters of the lesions; their abrupt commencements show the production of 8 mm. wheals by the original injection of tuberculin, and serve to indicate the vertical scale of the graphs. The heavy horizontal lines represent the actual time interval between irradiation and injection. Lesions already established before irradiation are unaffected, but there is a steadily increasing suppression of reactions begun after irradiation.

later, with massive lung tuberculosis. Of the two not irradiated, one was killed at four months and showed a few small tubercles in its lungs, and one is still alive at six months.

Histology of Tuberculin Reactions.

In three rabbits in which the tuberculin reaction was suppressed by irradiation, the histology of the sites of tuberculin injection during the negative phase was studied. Biopsies were made at varying intervals from 6–24 hr. after the injection of tuberculin. The six biopsies so obtained showed a consistent picture of a rapidly advancing polymorph exudate, predominantly perivascular (Fig. 3) but beginning to be confluent at 24hr. Both in volume of tissue affected and intensity of response there appeared to be no obvious difference between these lesions and those produced in non-irradiated animals. Companion tuberculin injections at other sites in the same animals showed the normal rapid disappearance of the original injection wheal, a transient erythema, and at 48 hr. a negative result. The contrast between macroscopic negativity and microscopic activity was a striking one. Saline injection or tuberculin injection into non-infected animals produced only slight transient microscopic reactions.

In view of Humphrey's (1951) findings on the effect of cortisone on the Arthus reaction, it was thought worth while to obtain a similar series of biopsies in cortisone-treated animals. After some preliminary experiments to find a suitable dose, 40 mg. was adopted as standard ; 25 mg. was injected into the thigh muscles 4–6 hr. before the tuberculin injection, and a further 15 mg. at the time of the tuberculin injection. No visible lesion was produced by the tuberculin in the rabbits used, but the cellular response, exactly as in the irradiated animals, appeared to be unaltered (Fig. 4).

Other findings with cortisone.

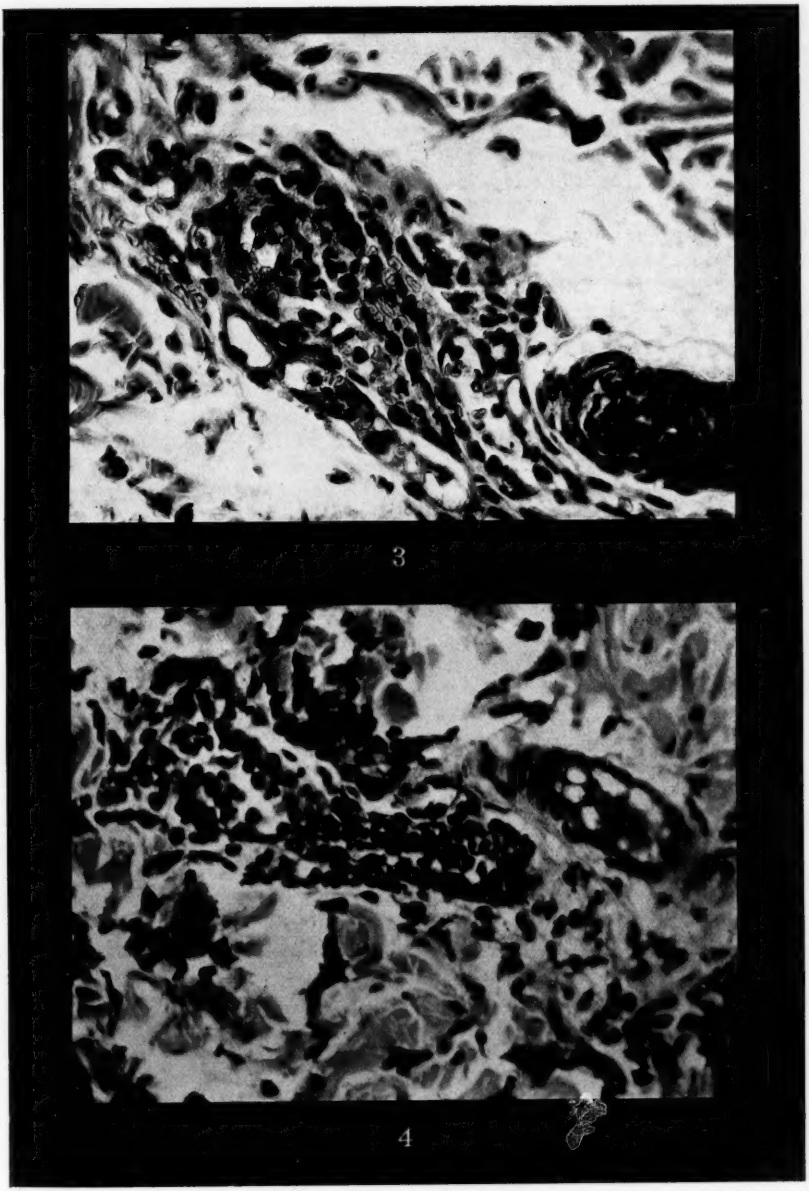
Our experiments with cortisone were designed only to serve as a basis for comparison with the effects of irradiation. It may, however, be said that cortisone in a dose of 40 mg. given as described was sufficient to produce a negative tuberculin reaction in most rabbits, and that the effect was the same whether the infecting organism was B.C.G. or human strain, or both. The effect of this 40-mg. dose appeared to be similar to that of 200 r of X rays, although of course the latter produces a much more prolonged negative phase.

Cortisone was given to 13 tuberculin-positive rabbits, 9 of which became negative after 40 mg. or less and all of which showed some reduction in reaction diameter. In 11, more than one dose level was used, and the results were sufficient to indicate that the response in any one rabbit was consistent, even after (in two cases) the lapse of several months. Twelve of these rabbits were later irradiated. In 9 of these the effect of the irradiation was the same as that of the cortisone (6 became negative with both and 3 remained positive with both) : in 3 the results

EXPLANATION OF PLATES.

FIG. 3.—Intradermal tuberculin reaction in an irradiated rabbit : perivascular polymorph infiltration in response to 1:100 old tuberculin given 18 hours earlier. No macroscopically visible or palpable induration was present.
Haemalum and eosin. × 200.

FIG. 4.—Intradermal tuberculin reaction in a rabbit receiving cortisone : an exactly similar response to that of Fig. 2.
Haemalum and eosin. × 200.



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Lennox, Dempster and Boag.

differed (2 remained positive with X rays and became negative with cortisone ; 1 reversed this). The degree of coincidence is below the level of statistical significance, but taken with other evidence may be regarded as suggestive of some common factor at work.

It would have been of great interest to determine whether the various factors shown (Long and Miles, 1950 ; Long, Miles and Perry, 1951 *a* and *b*) to influence the effect of cortisone on tuberculin sensitivity in guinea-pigs also applied to irradiation. Humphrey (personal communication to Long *et al.*, 1951*a*) has shown, however, that a cabbage-free diet does not modify the cortisone effect in rabbits. We were able to confirm that even by adding large amounts of ascorbic acid to the no-cabbage diet, and by giving thyroxine in high dosage (1 mg. on alternate days) with the cabbage diet, no demonstrable difference in the cortisone effect was produced. If the rabbit resembled the guinea-pig, the ascorbic acid/no-cabbage diet should have caused a considerable reduction in the cortisone effect ; cabbage/thyroxine an enhancement of it. Our groups consisted of only 5 rabbits each, but the effect of cortisone on the tuberculin reaction was tested in each animal with some care at several dose levels. In any case, only if the effect was a gross one was there any prospect of demonstrating a corresponding effect of irradiation without the expenditure of a large number of animals, so we did not pursue the matter further.

DISCUSSION.

The postponement effect, being exactly what was expected, need not be further considered here. The suppression effect, which appears in comparison with the effects of irradiation on other immune reactions to be highly anomalous, calls for an attempt at explanation. Three possible mechanisms suggest themselves :

(a) *Local irradiation effect.*—A local effect of the irradiation in the area of the reaction might *a priori* be supposed to be concerned. By screening the test area we have eliminated this.

(b) *Cellular destruction by irradiation.*—Cellular destruction produced by irradiation might be invoked, especially as lymphocytes are foremost amongst the cells affected. Lymphocytes are certainly among the cells of the sensitized individual which are most sensitive to tuberculin, and it has even been suggested (Favour, 1947 ; Fremont-Smith and Favour, 1948) that their lysis is always the primary event. Irradiation might be supposed to destroy a sufficient proportion of the tuberculin-sensitive cells of the body to interfere with the reaction. The principal evidence against this lies in our histological findings : the cellular reaction is unaffected. Even with profound lymphopenia which must have been present in our rabbits, a normal liberation of leukotaxic substance occurred ; sensitive cells (whether local fixed tissue cells or polymorphs) must have still been able to react with the tuberculin injected. It is perhaps fortunate that the normal cellular response in the tuberculin reaction is a polymorph one (Angevine and Seastone, 1950) ; without these relatively radio-resistant cells as indicators it might have been difficult to recognise that any reaction was still occurring. A less conclusive piece of evidence is to be found in the timing of the irradiation effect : cell destruction occurs in a few hours (Bloom, 1948), and any effect depending directly on it might be expected to be equally abrupt in onset.

(c) *An indirect adrenal-mediated mechanism.*—The evidence for the secretion of cortisone by the adrenals after irradiation is indirect but reasonably good.

Leblond and Segal's (1942) demonstration of atrophy of lymphoid tissue in areas remote from the actual site of irradiation, and of the abolition of this atrophy by adrenalectomy, is hard to explain in any other way; others who have confirmed this are Dougherty and White (1946), Gregoire (1943), and Halberstaedter and Ickowicz (1947). Patt, Swift, Tyree and John (1947) studied the weights and cholesterol contents of the adrenals of irradiated animals and their results strongly suggest hypersecretion: this last effect can be prevented by hypophysectomy (Patt, Swift, Tyree and Straube, 1948). Urinary 17 keto-steroids are increased after irradiation (Lawrence, 1949); we hope to be able to present more immediately relevant evidence on the subject of steroid excretion after irradiation later. There is some evidence also from each of these sources that secretion does not reach its maximum level until, at earliest, the second day, and, with doses of the order of those we have used, that it is continued for some time thereafter. The gradual onset of the suppression effect which is shown in Fig. 1 can be related to this.

Cortisone has been shown to suppress or weaken the tuberculin reaction in guinea-pigs (Long and Miles, 1950; Harris and Harris, 1950) and in man (Long and Favour, 1950). Negative results which have been reported (Sheldon, Cummings and Evans, 1950; Derbes, Dent, Weaver and Vaughan, 1950) probably only reflect the quantitative nature of the phenomenon, large doses of cortisone being needed to suppress the manifestation of a very active reaction. Our own experiments show that, at least in the rabbit, the strain of the infecting organism does not affect the result. Humphrey's demonstration (1951) of a similar effect of cortisone on the Arthus phenomenon is also relevant. Given, then, that total body irradiation almost certainly stimulates the secretion of cortisone, and that cortisone can certainly produce the effect we have demonstrated in the manner and at the times we have shown it to occur, it becomes reasonable to regard this as the mechanism involved.

We do not pretend to believe that we have produced a complete proof that this mechanism is wholly responsible for the effect. Direct proof by repetition of our experiments after adrenalectomy we have failed to obtain: none of our rabbits survived adrenalectomy (Vogt, 1943) longer than 24 hours. Hypophysectomy would also serve the same purpose, but this we have not attempted. At least, if our hypothesis is the right one we can cease to regard the response of the tuberculin reaction to irradiation as anomalous: the fundamental sensitivity of the tissues to tuberculin is unaltered; it is only its manifestation by macroscopically visible oedema which is suppressed by the steroid incidentally formed. Another consequence which must follow from the acceptance of our hypothesis is the necessity for re-examining other examples of the radiation modification of immune responses; there may be other cases in which the effect is an adrenal-mediated one.

SUMMARY.

Irradiation of a rabbit at or near the time of inoculation with tubercle bacilli delays the appearance of the positive intradermal tuberculin reaction.

Irradiation of the tuberculin-sensitive rabbit temporarily renders this reaction negative or markedly weaker.

Local screening does not alter this effect.

The histology of the tuberculin reaction sites, and the timing of the process,

suggest that the suppression is the result of overproduction of cortisone under the stimulus of irradiation.

Our thanks are due to Professor J. H. Dible and Dr. D. A. Mitchison for their advice and criticism, and to Sir Macfarlane Burnet for the original stimulus to these experiments. The P.P.D. used we obtained from the Ministry of Agriculture Veterinary Laboratory through the kindness of Dr. A. W. Stableforth, and most of the cortisone was provided from the generous gift made jointly to the Medical Research Council and the Nuffield Foundation by Messrs. Merck & Co., Inc. The histological sections are by Mr. J. J. Griffin and the photomicrographs by Mr. E. V. Willmott.

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TITRATION OF ANTISERA TO SOLUBLE PROTEINS ON THE BASIS OF AN AGGLUTINATION REACTION:

Conjugation of Egg Albumin and Chicken Serum Globulin to the Incomplete Rh Antibody and the Subsequent Use of Rh-Positive Cells, Sensitized By such Conjugated Incomplete Antibodies, to Titrate Antisera against Egg Albumin and Chicken Globulin.

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It has been recognised for many years that the agglutination reaction is much more sensitive than the precipitation reaction as a serological method for demonstrating the specific antibody content of a serum.

Arkwright (1914) showed that an apparent agglutination phenomenon was produced if he added to a non-precipitating mixture of a diluted watery extract of *Salm. typhi* and diluted specific anti-typhoid serum a suspension of an unrelated organism such as *Bact. coli*. He also mentioned earlier workers who had noticed the same phenomenon. Arkwright's interpretation seemed to be that during the union of antigen and antibody a web is formed which entangles the unrelated organism or other inert particles and causes them to clump together. Jones (1927), who made similar observations, gave a different interpretation in a paper entitled "Agglutination by precipitation." He demonstrated the agglutination of collodion particles coated with a protein antigen by its specific precipitin serum. That the antigen was adsorbed on to the surface of the particle could be shown by the fact that agglutination still occurred even if the coated particles had been washed before adding the precipitin serum. Jones as well as Arkwright commented on the greater sensitivity achieved by performing the reactions of a precipitin serum on the basis of an agglutination test.

Cannon and Marshall (1940) have further developed the "collodion particle technique" as a sensitive method for determining the antibody titre in specific anti-protein sera. The latest and probably the most sensitive method based on this principle of converting a precipitation into an agglutination test when dealing with protein antigens is that described by Boyden (1951). He showed that protein antigens could be adsorbed on to red cells which had been mildly treated with tannic acid and that such cells could be agglutinated subsequently by specific antisera.

In two previous papers (Coombs, Mynors and Weber, 1950; Coombs, Mynors and Wild, 1951) an agglutination technique has been described for titrating serum antibodies against determinant groups such as arsanilic and sulphanilic acid. In this technique the compound, after diazotization, is coupled on to the incomplete

* In receipt of a grant from the Norfolk Education Committee.

Rh antibody, and may thereby be carried on to the surface of human Rh–positive red cells by the specific affinity of the antibody. Red cells sensitized by the conjugated incomplete antibody are quite stable in suspension, but after being washed they may be specifically agglutinated by antisera to the chemical grouping.

The present paper extends this study to the titration of serum antibodies against soluble protein antigens. Experiments are described on the conjugation of egg albumin to the incomplete Rh antibody. Red cells sensitized with the conjugated antibody possess egg albumin receptors on their surface and are stable in suspension. After washing in saline such cells may be agglutinated specifically by anti-egg albumin serum and may be used to titrate the agglutinating strength of the serum. Chicken globulin has also been conjugated to the Rh antibody and has been used in similar tests.

In these early experiments the conjugation of two protein molecules has been achieved with tetrazotized benzidine. This compound has been used for coupling egg albumin directly to the intact red cell by Pressman, Campbell and Pauling (1942).

MATERIAL AND METHODS.

Egg albumin.

This was prepared by Hopkins' method described by Cole (1933). Once prepared, it was kept as an ammonium sulphate sludge at − 20° until required. The ammonium sulphate was removed by dialysis against saline.

Chicken serum globulin.

Chicken serum was heated for 30 min. at 54° and then absorbed free of agglutinins for human red cells. The globulins were precipitated by adding to the serum an equal volume of a saturated solution of ammonium sulphate. The precipitate was washed once with a half saturated solution of ammonium sulphate and then dissolved in saline. After dialysis to remove the ammonium sulphate a Kjeldahl estimation showed the protein concentration to be 2·5 per cent.

Rabbit antisera.

Anti-egg albumin sera.—The sera used were those of two rabbits which had received two and three courses respectively of intraperitoneal injections of egg albumin. Each course consisted of 4–5 injections of 0·5–2 ml. of a 1·25 per cent solution of egg albumin. The sera had been stored at − 20° without chemical preservative for 2 years.

Anti-chicken globulin serum.—The serum used was from a rabbit which had received two courses of intravenous injections of chicken serum. Each course consisted of 4–5 injections of 0·5–1 ml. chicken serum. This serum had been stored at − 20° without chemical preservative for 6 years.

Anti-human globulin serum.—This was prepared by injections of whole human serum as described by Coombs and Mourant (1947).

Sera withdrawn from these rabbits before injection were used for control purposes in the tests. All the sera were heated at 56° for 30 min. and absorbed free of agglutinins for human red cells.

Human sera containing incomplete Rh antibody.

Two human sera, both containing incomplete anti-D Rh antibodies, have been used. Incomplete antibodies combine specifically with the homologous cellular antigen, but for some reason, as yet not understood, fail to cause the cells to agglutinate. Normal human sera, both heated and unheated, containing no Rh antibodies have been used as controls.

Red cells.

Blood was obtained either by venepuncture and mixing with Wintrobe's oxalate mixture, or by an ear-prick and collecting the drops of blood in 0·6 per cent sodium citrate in isotonic

saline. Group O Rh D positive and O Rh D negative bloods were used. The red cells were washed three times in saline before use.

Preparation of tetrazotized benzidine.

A solution of benzidine tetrazonium chloride was prepared as follows: 23 mg. benzidine (1 mol.) was dissolved in 7·5 ml. N/10 HCl (6 mols.) and the solution cooled in an ice-bath to below 5°; 1·25 ml. N/5 freshly prepared sodium nitrite solution (2 mols.) was added slowly with stirring and the solution allowed to stand at least 10 min. A faint yellow coloration appeared.

The total volume, 8·75 ml., contained sufficient tetrazotized benzidine for 100 ml. of a 5 per cent egg albumin solution on the basis of 1 mole benzidine tetrazonium chloride per mole of protein, assuming the molecular weight of egg albumin to be 40,000.

Conjugation of egg albumin with the protein molecules of an incomplete Rh antiserum.

To obtain a conjugation on the calculated basis of 8 moles benzidine tetrazonium chloride per 2 moles of protein, 0·7 ml. of the above tetrazotized benzidine solution was added slowly in the cold to an approximately equimolecular mixture of 2·5 ml. Rh antiserum (assumed 5 per cent protein of average molecular weight 100,000) and 1 ml. 5 per cent egg albumin solution.

After standing at 5° for 10 min., the pH of the solution was adjusted to 7·2 with sodium bicarbonate and the serum left at 4° overnight. The following day the conjugated serum was dialysed against saline at 4° for 24 hr. The conjugated serum was then recovered and placed at − 20° until required for testing. There seems no reason why the conjugated serum could not be stored indefinitely under such conditions.

Egg albumin was also conjugated with the proteins of normal serum under identical conditions.

Conjugation of chicken serum globulin with the protein molecules of an incomplete Rh antiserum.

The procedure here was similar to that already described for the conjugation of egg albumin to the serum proteins: 0·35 ml. of the tetrazotized benzidine solution was added in the cold to an approximately equimolecular mixture of 1·25 ml. Rh serum and 3·0 ml. 2·5 per cent solution of chicken globulin. The serum mixture was neutralised, dialysed and stored as before.

EXPERIMENTAL.

Titration of incomplete Rh antibody coupled with egg albumin against Rh–positive cells and the subsequent detection of the sensitization with anti-egg albumin and anti-human globulin rabbit serum.

Four samples of incomplete Rh antiserum were conjugated with egg albumin on the calculated basis of 4, 8, 12 and 16 moles of tetrazotized benzidine per 2 moles protein respectively.

Titrations of the four conjugated antisera were set up in 0·25 ml. amounts and an equal volume of a 2 per cent suspension of Group O Rh–positive human red cells added. After incubation for 1 hr. at 37° the cells were centrifuged, washed twice in saline and re-suspended in 0·25 ml. saline, The cells were then tested for sensitization by the conjugated antibody by adding a volume of each suspension to equal volumes of selected dilutions of a rabbit anti-egg albumin serum and a rabbit anti-human globulin serum. A dilution of normal rabbit serum served as a control. These last three sera had previously been absorbed free of agglutinins for human red cells. The mixtures were incubated for a further 1 hr. at 37° and the sedimented cells read for agglutination.

The anti-Rh serum conjugated with egg albumin on the calculated basis of 8 moles of tetrazotized benzidine per 2 moles protein gave the best results, and these are shown in Table I. With fewer groups of tetrazotized benzidine there

appeared to be much Rh antibody unconjugated, but with 12 and 16 moles the combining affinity of the Rh antiserum was diminished.

TABLE I.—*Titration of Incomplete Rh Antibody Coupled with Egg Albumin against Rh-positive Cells and the Subsequent Detection of the Sensitization with Anti-Egg Albumin and Anti-Human Globulin Rabbit Serum.*

Antisera used for detecting sensitization.		Sensitizing system : dilutions of conjugated Rh anti-serum.									
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Saline.
Normal rabbit serum	1:40	−	−	−	−	−	−	−	−	−	−
Rabbit 53 2nd course anti-egg albumin	1:10	++	++	++	−	−	−	−	−	−	−
Rabbit 53 2nd course anti-egg albumin	1:40	++	++	++	++	++	−	−	−	−	−
Rabbit 53 2nd course anti-egg albumin	1:640	++	++	++	++	++	+	−	−	−	−
Rabbit 9A anti-human globulin	1:40	++	++	++	++	++	++	++	+	−	−

++ = strong agglutination.
+ = definite, but weak, agglutination.
− = no agglutination.

A 2 per cent suspension of Rh-positive cells was added to a titration of incomplete Rh anti-serum conjugated* with egg albumin. After a period for sensitization the cells were washed in saline and volumes of each suspension added to equal volumes of anti-serum (first column).

* Conjugation on a calculated basis of 8 moles tetrazotized benzidine per 2 moles protein.

To show that the egg albumin protein was adsorbed on the cell surface solely by its attachment to the incomplete Rh antibody, the following further controls were performed : (1) Rh-positive cells were exposed to a simple mixture of Rh serum and egg albumin. After sensitization and washing, the cells were agglutinated only by the anti-human globulin serum and were quite stable in the anti-egg albumin serum. (2) Rh-negative cells exposed to the conjugated anti-Rh serum were agglutinated neither by the anti-human globulin serum nor by the anti-egg albumin serum. (3) Rh-positive cells previously exposed to a normal human serum conjugated with egg albumin were also unagglutinated.

These results show that cells sentitized with incomplete Rh antibody conjugated with egg albumin are stable and remain unagglutinated in normal rabbit serum, but may be agglutinated specifically by anti-egg albumin serum.

Titration of the pre- and post-inoculation sera of two rabbits, inoculated with egg albumin, for their agglutinating antibody content against egg albumin.

The pre- and post-inoculation sera of two rabbits inoculated with egg albumin were titrated in 0·1 ml. amounts. These sera had previously been absorbed free of agglutinins for human red cells. One-drop volumes of each serum dilution were placed in small tubes in which the tests were performed.

The cell suspension possessing egg albumin receptors to be added to the serum titrations was prepared as follows : 2·5 ml. of a 2 per cent suspension of Group O Rh-positive human red cells were added to an equal volume of a $\frac{1}{4}$ dilution of an incomplete Rh antiserum previously conjugated with egg albumin (calculated 8 moles tetrazotized benzidine per 2 moles protein). A similar mixture was set up using Rh-negative cells to serve as a control. After the mixtures had stood for 1 hr. at 37° the cells were centrifuged down, washed twice and re-suspended in saline to a 2 per cent suspension.

One drop of the treated Rh-positive cell suspension was added to each tube of the serum titrations and the treated Rh-negative cells were added to duplicate serum titrations. The tubes were again incubated at 37°, and after 1 hr. the sedimented cells examined for agglutination.

The results are recorded in Table II.

TABLE II.—*Titration of the Pre- and Post-Inoculation Sera of Two Rabbits Inoculated with Egg Albumin for their Agglutinating Antibody Content against Egg Albumin.*

Sera* titrated.	Cells carrying egg albumin antigen.	Titre of serum producing agglutination.
R. 53 Pre-inoculation	Rh-positive cells exposed to incomplete Rh antibody conjugated with egg albumin and subsequently washed	0
R. 52 Pre-inoculation		0
R. 53 2nd course anti-egg albumin		40,000
R. 52 3rd course anti-egg albumin		160,000
R. 52 Pre-inoculation	Rh-negative cells similarly treated	0
R. 52 3rd course anti-egg albumin		0

* All sera previously absorbed free of agglutinins to human red cells.

Inhibition by a solution of egg albumin, of the agglutinating action of anti-egg albumin serum on red cells carrying egg albumin receptors.

The agglutinable red cell suspension carrying egg albumin receptors was prepared as described in the first experiment.

One drop of an anti-egg albumin serum (R. 53 2nd course) diluted 1/40 was placed in each tube of three series of 20 tubes. A similar set of three series of tubes was set up with one drop of the same serum diluted 1/640. One drop of a serial dilution of human serum (free of iso-agglutinins for human cells) was added to the tubes of the first series of each set, beginning with the greatest dilution of the serum. Likewise, a drop of a serial dilution of chicken serum (also agglutinin-free) and of a serial dilution of a 5 per cent egg albumin saline solution was added to the tubes of the second and third series of each set respectively. The contents of the tubes were mixed and placed at 37° for 30 min. to allow for neutralization of the antiserum. One drop of the agglutinable cell suspension carrying egg albumin receptors was then added to all the tubes. After 1 hr. at 37° the cells in each tube were read for agglutination.

The results are shown in Table III. Only the egg albumin solution inhibited the agglutinating action of the anti-egg albumin serum on the human cells carrying egg albumin receptors.

TABLE III.—*Inhibition by a Solution of Egg Albumin of the Agglutinating Action of Anti-Egg Albumin Serum on Red Cells Carrying Egg Albumin Receptors.*

Protein solutions* for inhibition test.	Highest dilution of protein solution inhibiting the agglutinating action of rabbit anti-egg albumin serum, diluted:	
	1/40	1/640
Human serum	0	0
Chicken serum	0	0
5 per cent solution of egg albumin	1:5,120	1:320,000

* A 1/5 dilution was the first dilution tested.

Agglutinable cells: Rh-positive cells sensitized with incomplete Rh antibody conjugated with egg albumin and subsequently washed.

The conjugation of chicken serum globulin to the serum proteins of an incomplete Rh antiserum and the agglutination of Rh-positive red cells, sensitized by such a conjugated serum and subsequently washed, by a rabbit anti-chicken globulin serum.

The conjugation between chicken serum globulin proteins (absorbed free of antibodies to human cells) and the proteins in an incomplete Rh antiserum was accomplished as described in "Methods." An experiment was then carried out with this conjugated antiserum similar to that performed with the serum conjugated with egg albumin.

The anti-Rh serum conjugated with chicken globulin was titrated in 0·25 ml. amounts. To each tube was added 0·25 ml. of a 2 per cent Group O Rh-positive cell suspension. After incubation for 1 hr. at 37° the cells were centrifuged down, washed twice in saline and re-suspended as a stable cell suspension in 0·25 ml. saline. One-drop amounts from each tube of the titration were then added to fresh tubes already containing one-drop amounts of chosen dilutions of normal rabbit serum, rabbit anti-egg albumin serum, rabbit anti-chicken globulin serum and rabbit anti-human globulin serum. These sera had been previously absorbed free of agglutinins to human red cells.

The contents of the tubes were mixed and placed at 37° for the cells to sediment. The resulting agglutination is recorded in Table IV. On performing a similar test

TABLE IV.—*Titration of Incomplete Rh Antibody Coupled with Chicken Globulin against Rh-positive Cells and the Subsequent Detection of the Sensitization with Anti-Chicken Globulin and Anti-Human Globulin Rabbit Serum.*

Antisera used for detecting sensitization.		Sensitizing system : dilutions of conjugated anti-Rh serum.									
		1:3	1:6	1:12	1:24	1:48	1:96	1:192	1:384	1:768	Saline
Normal rabbit serum	1:40	−	−	−	−	−	−	−	−	−	−
R. 52 3rd course anti-egg albumin	1:40	−	−	−	−	−	−	−	−	−	−
R. 43 2nd course anti-chicken serum	1:10	++	++	++	++	−	−	−	−	−	−
R. 43 2nd course anti-chicken serum	1:40	++	++	++	++	+	−	−	−	−	−
R. 43 2nd course anti-chicken serum	1:640	++	++	++	++	+	−	−	−	−	−
R. 9A anti-human globulin	1:40	++	++	++	++	++	++	++	+	−	−

Key as in Table I.

A 2 per cent suspension of Rh-positive cells was added to a titration of incomplete Rh anti-serum conjugated with chicken globulin. After a period for sensitization the cells were washed in saline and volumes of each suspension added to equal volumes of anti-serum (first column).

with Rh-negative instead of Rh-positive cells no agglutination resulted. This indicated that the chicken globulin receptors were carried to the cell surface solely by their attachment to the incomplete Rh antibodies.

From Table IV it may be seen that the anti-egg albumin serum had no agglutinating action on cells possessing chicken serum globulin receptors. It may also be stated that the anti-chicken globulin serum had no agglutinating action on human cells sensitized with unconjugated incomplete Rh antibody.

The rabbit anti-chicken globulin serum used in these tests was a very weak precipitin serum giving only a very doubtful interfacial test with the chicken serum

antigen diluted 1 : 1000. It had been prepared six years previously and since stored at −20°. On titrating its agglutinating strength by the present method, its anti-chicken globulin titre was found to be 1280. The interaction between one volume of a cell suspension carrying chicken globulin receptors and one volume of the anti-chicken globulin serum diluted 1 : 320 (i.e., 4 agglutinating doses) was not inhibited by one volume of a 1 : 10 dilution of human serum nor by a similar dilution of a 5 per cent solution of egg albumin. The reaction was, however, inhibited by one volume of a 1 : 40,000 dilution of chicken serum.

DISCUSSION.

The object underlying the development of the technique described in this paper was to find a means of detecting incomplete non-precipitating (or non-agglutinating) antibodies reacting with soluble protein antigens or polypeptide haptens.

Boyden's (1951) method of adsorbing soluble protein antigens on to tanned cells, a development of the "antigen coated particle technique," is probably one of the most sensitive methods for detecting potentially agglutinating antibodies to soluble protein antigens. By potentially agglutinating antibodies is meant antibodies which will produce agglutination if the antigen is fixed on a surface. However, there is already much accumulated evidence that there exist antibodies to soluble proteins which for some reason, as yet unknown, are non-precipitating and which may be presumed also to lack the property of producing agglutination of particles carrying the homologous antigen on their surface. Unfortunately, the possibility of demonstrating this latter type of "incomplete" antibody on tanned cells previously coated with antigen seems to be excluded because normal serum globulin is also adsorbed on to such treated cells and the use of the antiglobulin reaction is thus precluded.

With the technique described in the present paper the surface of the red cell is modified in no way other than the adsorption at particular specific sites of the conjugated antibody which carries the new protein antigen receptor to the cell surface. In theory, this technique could be developed so as to allow the final criterion of the combination of the antibody to the protein receptor to be judged by an anti-globulin reaction. As yet we have not sought incomplete antibodies to soluble protein antigens by this method. For this development it would be desirable to have the soluble protein antigen conjugated to a non-agglutinating rabbit antibody rather than a human antibody so that the coupling antibody would be of the same species nature as the rabbit anti-globulin serum. This paper, therefore, has been confined to the demonstration of potentially agglutinating antibodies to soluble protein antigens, and for these tests the incomplete Rh antibody has proved ideal as a carrier for the new receptor.

We are by no means satisfied with the process of conjugation adopted in these early experiments. In the first place, the combination of the protein molecules is random, and presumably many Rh antibody molecules combine with their own serum proteins instead of with egg albumin molecules. Also working with the whole antiserum, instead of a more pure antibody preparation, is very wasteful of the protein antigen to be conjugated. There is also the added possibility that the introduced tetrazotized benzidine may, under certain circumstances, confer a new specificity on the conjugated protein antigen. Nevertheless, the results obtained so far have been encouraging and serve to illustrate the principle of the method.

The incomplete Rh antibody is admirably suited for carrying the conjugated protein antigen, as cells sensitized with it are absolutely stable in saline or dilutions of normal sera. However, for the further development of the work, especially in applying it to the search for incomplete antibodies to protein antigens, it would be desirable to conjugate the protein antigen to a non-agglutinating rabbit anti-red cell antibody. Work on such a system is under way.

Work is also in progress on conjugating polysaccharides to non-agglutinating red cell sensitizing antibodies to allow the application of the method to the detection of antibodies specific for polysaccharide antigens or haptens.

Finally it may be added that these preliminary experiments have been performed with egg albumin and chicken serum globulin merely to show the principle of the method. It is intended to apply the technique to investigations with proteins, polypeptides and polysaccharides of biological and pathological importance especially in the field of allergy.

SUMMARY.

A technique has been described for titrating antisera against soluble protein antigens on the basis of an agglutination reaction.

The method involves the conjugation of the protein antigen with the incomplete Rh antibody. The anti-protein serum may then be titrated against red cells sensitized with the incomplete Rh antibody conjugated with the protein concerned.

Using this method rabbit antisera against egg albumin and chicken serum globulin respectively have been titrated. Inhibition tests with very dilute solutions of the respective antigens confirmed the specificity of the reactions.

Implications of the method have been discussed. By its application it should be possible to measure incomplete or non-precipitating antibodies to soluble protein antigens. It is intended to apply the method to investigations on allergy.

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THE EFFECT OF 3:5-DINITRO-ORTHO-CRESOL ON THE ORGANIC PHOSPHATES OF MUSCLE.

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ALTHOUGH shock is characterized by a great fall in energy production, the total body hexose disappears as rapidly as in the normal animal, despite the lower body temperature of the shocked animal (Stoner, Threlfall and Green, 1952). A possible explanation of this apparent discrepancy is the uncoupling of phosphorylation from oxidation in the tricarboxylic acid cycle. This can be achieved *in vitro* by a wide range of compounds but, *in vivo*, many of them appear inert, whilst others are slow to exert their full effect. From the recent studies of Bidstrup and Payne (1951) and Parker, Barnes and Denz (1951), the insecticide 3:5-dinitro-ortho-cresol (DNOC) would seem an ideal substance for studying the biochemical consequences of rapid uncoupling in the whole animal. A feature of poisoning by this compound, in addition to pyrexia and increased O_2 consumption, is the appearance of rigor just before death. As Parker *et al.* (1951) have suggested, this may be due to the disappearance of high energy phosphate bonds ($\sim$ P) from the muscle since Bate-Smith and Bendall (1947, 1949) and Bendall (1951) have shown that this precedes *rigor mortis*. In studying the tissue changes in rapid uncoupling we have sought to substantiate this explanation of the rigor of DNOC poisoning.

METHODS.

Forty-seven fasting male albino rats were used. DNOC was purified and prepared for injection, in 0·1 M Na_2CO_3, as described by Parker *et al.* (1951). Carrier-free ^{32}P was obtained from the Atomic Energy Research Establishment, Harwell, and diluted (10 ml. per mC.) with phosphate-saline buffer, pH 7·0 (Harrison and Raymond, 1951). This solution was assayed, after an aliquot had been diluted and plated out on stainless steel planchettes, with an end-window counter, the geometry of which had been previously determined with a standard sample of ^{32}P. The ^{32}P (25μ C. per 100 g. body wt.) was given intramuscularly in the right hind limb at a time when the rat showed signs of poisoning. The dose of ^{32}P, containing less than 1·0 mg. carrier P, was measured and injected as quickly as possible (Lamerton and Harriss, 1951).

DNOC-treated rats were usually sampled at death, and controls anaesthetized with nembutal (5 mg. per 100 g. body wt. intraperitoneally) and treated with 15 mg. α-β-dihydroxy-γ-(2-methyl phenoxy)- propane (" myanesin "—B.D.H.) per 100 g. body wt. intraperitoneally, were killed at a similar interval after the ^{32}P injection, the experiments being done in pairs. We have confirmed the work of Bate-Smith and Bendall (1949) on the value of "myanesin " when seeking the true resting values of certain labile muscle constituents.

Plasma was obtained from heparinized heart blood. After deproteinization with 9 vols. 10 per cent trichloroacetic acid (TCA) the inorganic P was precipitated as Mg NH_4PO_4. Sacks' (1949) magnesia mixture was used, 24–48 hr. at 0° being allowed for precipitation and the precipitate was washed twice with 10 per cent. (v/v) NH_4OH before estimation.

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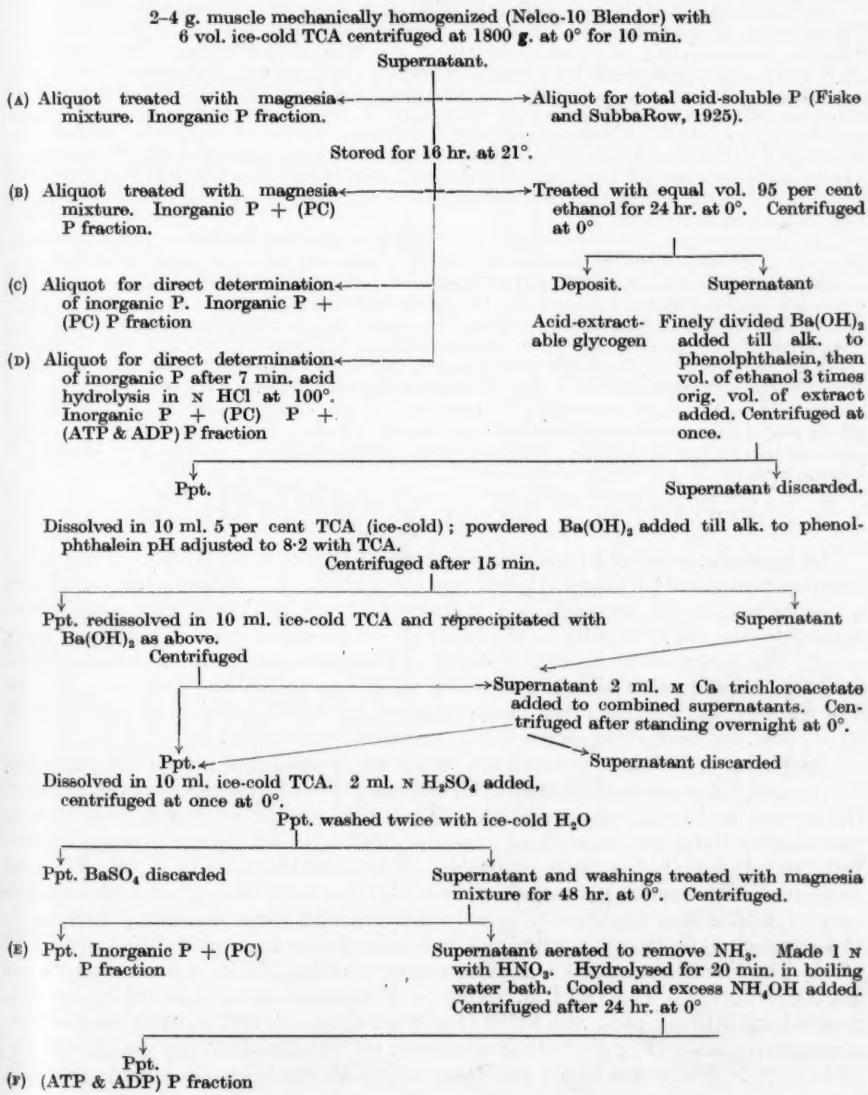
Muscle from the left hind-limb was frozen in liquid air after being rapidly freed of fat, nerves, etc., and treated as shown in Table I. This scheme combines previous methods (Stoner, 1950) with those of Umbreit, Burris and Stauffer (1945) and Sacks (1949). The acid-soluble P was completely extracted during homogenization. No carrier P was added

TABLE I.—*Scheme for the Fractionation of Some of the Phosphate Compounds of Muscle.*


2–4 g. muscle mechanically homogenized (Nelco-10 Blendor) with 6 vol. ice-cold TCA centrifuged at 1800 g. at 0° for 10 min.
Supernatant.
(A) Aliquot treated with magnesia mixture. Inorganic P fraction.
Aliquot for total acid-soluble P (Fiske and SubbaRow, 1925).
Stored for 16 hr. at 21°.
(B) Aliquot treated with magnesia mixture. Inorganic P + (PC) P fraction.
Treated with equal vol. 95 per cent ethanol for 24 hr. at 0°. Centrifuged at 0°
(C) Aliquot for direct determination of inorganic P. Inorganic P + (PC) P fraction
Deposit.
Supernatant
(D) Aliquot for direct determination of inorganic P after 7 min. acid hydrolysis in N HCl at 100°. Inorganic P + (PC) P + (ATP & ADP) P fraction
Acid-extractable glycogen
Finely divided $Ba(OH)_2$ added till alk. to phenolphthalein, then vol. of ethanol 3 times orig. vol. of extract added. Centrifuged at once.
Ppt.
Supernatant discarded.
Dissolved in 10 ml. 5 per cent TCA (ice-cold); powdered $Ba(OH)_2$ added till alk. to phenolphthalein pH adjusted to 8·2 with TCA.
Centrifuged after 15 min.
Ppt. redissolved in 10 ml. ice-cold TCA and reprecipitated with $Ba(OH)_2$ as above.
Supernatant
Centrifuged
Supernatant 2 ml. M Ca trichloroacetate added to combined supernatants. Centrifuged after standing overnight at 0°.
Ppt.
Supernatant discarded
Dissolved in 10 ml. ice-cold TCA. 2 ml. N H_2SO_4 added, centrifuged at once at 0°.
Ppt. washed twice with ice-cold H_2O
Ppt. $BaSO_4$ discarded
Supernatant and washings treated with magnesia mixture for 48 hr. at 0°. Centrifuged.
(E) Ppt. Inorganic P + (PC) P fraction
Supernatant aerated to remove NH_3. Made 1 N with HNO_3. Hydrolysed for 20 min. in boiling water bath. Cooled and excess NH_4OH added. Centrifuged after 24 hr. at 0°
Ppt.
(F) (ATP & ADP) P fraction

and the magnesia mixture was used as above. Phosphate was determined by the method of Fiske and SubbaRow (1925), using a Spekker photometer, except in the case of the isobutanol extracts, when the method of Berenblum and Chain (1938) was used. The muscle water content was taken as 75 per cent, the average value in our normal rats, which was not altered after DNOC treatment. The radioactivity of the solutions used for P estimation was determined with a liquid counter (Veall, 1948) recording the disintegrations in the usual way. The radioactivity of the muscle fractions, corrected for decay from the time of injection, is expressed as counts per min. per mg. P (specific activity).

The precipitation of the inorganic P in fractions A and B by the magnesia mixture appeared complete, since there was good agreement between the P in these fractions and that found in corresponding isobutanol extracts of the TCA extract (Ennor and Rosenberg, 1952) and between the results for fractions B and C. By using small aliquots (0·5 ml.) and not adding carrier P, the size of the Mg NH_4PO_4 precipitate was kept below the level at which significant co-precipitation and/or adsorption of organic polyphosphates occurs (Ennor and Rosenberg, 1952). Good agreement was obtained between the specific activities of fractions A and B and those of the corresponding isobutanol extracts of the TCA extract. Agreement between the P content and specific activity of fractions B and E was also good, although the size of the Mg NH_4PO_4 precipitate in the latter case could lead to adsorption and co-precipitation errors.

The concentration and specific activity of the phosphocreatine (PC) $\sim$ P was obtained from the difference between fractions A and B. Similarly, the concentration of the $\sim$ P of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) was obtained from the difference between fractions C and D. We agree with Bendall (1951) that the contribution of other acid labile phosphates to fraction D is negligible in voluntary muscle. The concentration of $\sim$ P, so determined, is about twice that found in fraction F. Our values for (D − C) are similar to Bendall's (1951) and those for F somewhat lower than LePage's (1948). The full explanation of the discrepancy between fractions (D − C) and F is not known. Whilst part of it is due to adsorption on the $BaSO_4$ precipitate, other factors which may be concerned are adsorption on the Mg NH_4PO_4 of fraction E and incomplete precipitation in the final stage. Because of its purity, fraction F was used to obtain the specific activity of $\sim$ P in the nucleotide.

RESULTS.

Our general observations confirmed those of Parker *et al.* (1951). Their LD_{50} (subcutaneous) was 2·46 mg. DNOC per 100 g. body wt. and the compound had a similar toxicity in our animals. A dose of 2·3 mg. per 100 g. body wt. subcutaneously was either rapidly fatal (Table II), or produced signs of poisoning from which the rat recovered fairly quickly. Delayed deaths do not occur after DNOC. It was more effective when given intraperitoneally; after 5·0 mg. per 100 g. body wt. by this route death occurred in about 3 min. and the survival time could not be further shortened by increasing the dose.

As Parker *et al.* (1951) pointed out, in the rat, death is not always accompanied by pyrexia, but a constant feature of all degrees of DNOC poisoning was an increase in the rate and depth of respiration. When death occurred it was immediately preceded by the onset of a striking muscular rigidity so that *rigor mortis* was almost fully developed at the time of death. When DNOC muscle (Table II) was compared with normal rat muscle (Table III) the $\sim$ P associated with creatine and nucleotide was found to be greatly reduced with a corresponding increase in the inorganic P fraction but without significant change in the total acid soluble P level. The loss of nucleotide $\sim$ P, after treatment with DNOC, was also seen in the Ba fractionation. The (ATP & ADP) $\sim$ P concentration, determined by this method was 0·10 mg. per g. wet wt. (S.D. 0·08) in the muscle of the DNOC-treated rat, significantly lower ($P < 0·01$) than in normal rat muscle—0·25 mg. per g. wet wt. (S.D. 0·07). There was also a complete loss of acid-extractable muscle glycogen

TABLE II.—*The Effect of the Subcutaneous Injection of 2·3 mg. DNOC per 100 g. Body wt. on the Plasma Inorganic P and Muscle Phosphate Fractions of the Rat.* ^{32}P *(25μ C. per 100 g. Body wt.) given Intramuscularly 30 min. after the DNOC Injection.*

Rat No.	Survival time after ^{32}P injection (min.).	Plasma inorganic P.		Muscle.							
				Total acid-soluble P.		Inorganic P.		(PC) P.		(ATP & ADP) P.	
		mg. per 100 ml.	Specific activity.	mg./g. wet wt.	Specific activity.	mg./g. wet wt.	Specific activity.	mg./g. wet wt.	Specific activity.	mg./g. wet wt.	Specific activity.
1A	25	15·40	473000	1·72	14500	0·90	20700	0·06	11500	0·16	7890
2A	50	11·78	300000	1·72	13500	1·19	15900	0	—	0·01	7200
3A	35	15·38	518000	1·77	10350	0·92	14750	0	—	0·18	4020
4A	45	12·38	417000	1·65	13700	1·01	16630	0·27	6130	0·20	7350
5A	45	—	—	1·85	10300	1·22	13500	0·02	5000	0·21	7580
6A	100	9·55	199000	1·67	12000	1·14	14750	0·08	20500	0·15	10850
7A	30	16·50	552000	1·67	8240	1·08	10990	0	—	0·19	3400
8A	Mean	13·50*	410000	1·71	11800†	1·07*	15320	0·06*	10780	0·16*	6900

* = Difference from control mean (Table III) significant at $P < 0·01$.
† = " " " " $P < 0·05$.

TABLE III.—*The Plasma Inorganic P and Phosphate Fractions of the Muscle of Normal Rats Killed under Nembutal and "Myanesin" at Varying Intervals after the Intramuscular Injection of 25μ C.* ^{32}P *per 100 g. Body wt.*

Rat No.	Interval after ^{32}P injection (min.).	Plasma inorganic P.		Muscle.							
				Total acid-soluble P.		Inorganic P.		(PC) P.		(ATP & ADP) P.	
		mg. per 100 ml.	Specific activity.	mg./g. wet wt.	Specific activity.	mg./g. wet wt.	Specific activity.	mg./g. wet wt.	Specific activity.	mg./g. wet. wt.	Specific activity
1B	30	6·19	572000	1·78	10500	0·44	15800	0·44	12750	0·43	7180
2B	55	5·59	423000	1·76	11400	0·48	15900	0·74	11600	0·46	10600
3B	35	6·25	423000	1·78	8600	0·48	10450	0·69	8220	0·45	6550
4B	45	7·18	438000	1·77	8450	0·23	21300	0·93	7750	0·48	6400
5B	45	—	—	1·85	7200	0·23	21400	0·82	6100	0·50	5900
6B	70	—	—	1·87	8430	0·32	14400	0·65	9590	0·51	7420
7B	100	7·15	230000	1·67	12700	0·26	20550	0·68	15900	0·43	12500
8B	30	7·15	479000	1·69	7780	0·29	18050	0·63	8800	0·42	5220
	Mean	6·59	428000	1·77	9380	0·34	17230	0·70	10090	0·46	7720

and a significant ($P < 0.01$) increase in the inorganic P of the plasma of the affected rat (Table II).

The picture seen in Table II is that found at death. Animals killed at the height of non-fatal poisoning only showed a fall in the acid-extractable muscle glycogen content and a slight rise in the plasma inorganic P.

Despite the striking changes in their concentration in fatal DNOC poisoning the specific activity of the inorganic P, (PC) ~ P and (ATP & ADP) ~ P of the muscle remained unaltered, as did that of the plasma inorganic P. The specific activity of the total acid-soluble P in the muscle of the treated rats was somewhat higher ($P < 0.05$) than in the controls both on comparison of means (t test) and on calculation of the variance ratio from the differences between the values in the DNOC-treated rats and their paired controls (Stoner, 1950).

DISCUSSION.

In fatal DNOC poisoning the muscle in rigor contains very much less (PC) ~ P and (ATP & ADP) ~ P than normal, the concentrations being reduced by 92 and 65 per cent respectively. The rise in the muscle inorganic P indicates that this is due to their local breakdown. The increase in inorganic P accounts for 78 per cent of the ~ P lost by these compounds. The remainder is probably distributed between the plasma and the acid-stable ester P fraction of the muscle. Not only are the immediate energy stores reduced but there is also complete destruction of the metabolically available muscle glycogen. This muscle closely resembles that in *rigor mortis*, where the glycogen reserve also disappears, and Bendall (1951) has shown that when the minimum length has been reached by muscle undergoing post-mortem shortening at 37°, the PC is almost completely hydrolysed and only 20 per cent of the (ATP & ADP) ~ P remains. In Bendall's experiments, changes of the magnitude recorded in ours were found when the shortening was 70 per cent of the maximum. The rigor of DNOC poisoning, therefore, is also probably due to physical changes in the contractile protein consequent upon the fall in nucleotide ~ P concentration (Szent-Györgyi, 1947). The main synthesis of these high energy bonds takes place in the tricarboxylic acid cycle. This stops after death through lack of O_2 and after DNOC through the uncoupling of phosphorylation from oxidation (Parker *et al.*, 1951). In both cases the tissue must then obtain its energy from glycolysis and this is usually insufficient for its needs, so that when the carbohydrate stores are exhausted the tissue stores of ~ P rapidly dwindle to produce the changes described in the muscle.

If this is the mechanism of DNOC poisoning it is not surprising that the specific activity of ~ P remains unaltered. From the Lohmann equation PC will reflect the activity of the ATP. As the specific activity of the inorganic P is unchanged, any ATP formed in the tricarboxylic acid cycle before complete uncoupling will contain the same proportion of ^{32}P as in normal muscle. After complete uncoupling, when ~ P is being formed during glycogenolysis, the ultimate source of P is still the inorganic P fraction through the formation of glucose-1-phosphate. The reason for the slight increase in the specific activity of the total acid-soluble P is not revealed by our experiments.

The close proximity to death of these muscle changes suggests that a critical level of DNOC is reached in all the tissues about the same time. The minimum

survival time of 3 min. after a maximal dose suggests, also, that once uncoupling is complete the energy stores will not support life for very long. This survival time is of the order to be expected from the estimates of the turnover of nucleotide ~ P given by Kalckar, Dehlinger and Mehler (1944) and Bendall (1951) and the amount present in such tissues as cardiac muscle (Davies, Francis and Stoner, 1947).

Although the muscle changes occur so very shortly before death they are specific. Changes of this magnitude would not occur for several hours if the limb was made ischaemic by the application of a tourniquet or if the animal was simply killed and *rigor mortis* allowed to develop. As we shall show, in a subsequent paper, when death occurs in shock produced by the injection of ATP there is no change in the ~ P level of the muscle although its specific activity is very much less than normal and the glycogen stores are very low. Findings, similar to those in nucleotide shock, have been obtained in muscle outside the injured area in shock following limb ischaemia (Bollman and Flock, 1945; Goranson, Hamilton and Haist, 1948). The changes of shock are, therefore, very different from those of uncoupling, and our present results help to elucidate the problem posed in our opening sentences.

SUMMARY.

The chemical changes associated with the rigor of 3:5-dinitro-ortho-cresol poisoning have been examined.

It was found that the concentrations of phosphocreatine, adenosine polyphosphate and glycogen were greatly reduced in the muscle which had developed rigor. Despite this the distribution of ^{32}P in the compounds containing high energy phosphate bonds was unaltered.

The rigor is considered to be due to the fall in the concentration of the high energy phosphate bonds in the muscle due to the uncoupling of phosphorylation from oxidation produced by 3:5-dinitro-ortho-cresol.

These results are discussed in relation to the energy changes in shock.

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THE PERSISTENCE OF RESISTANCE FOLLOWING THE DISAPPEARANCE OF HYPERSENSITIVITY IN GUINEA-PIGS AFTER VACCINATION WITH VOLE BACILLUS AND B.C.G.

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VACCINATION with B.C.G. has long been known to increase resistance to subsequent infection with virulent human or bovine tubercle bacilli in guinea-pigs (Calmette, Boquet and Nègre, 1924; Birkhaug, 1932; Schwabacher and Wilson, 1938). Wells and Brooke (1940) in a series of protection experiments found that vaccination with vole bacillus likewise increased the resistance of subsequently challenged guinea-pigs, although much smaller doses conferred a materially higher degree of protection over the same period of time, as judged from the extent of the tuberculosis found at autopsy eleven weeks after infection. The superiority in this respect of the vole bacillus over B.C.G. has been confirmed in cattle by several workers (Griffith and Dalling, 1940; Young and Paterson, 1949).

In most published work, challenging after vaccination with virulent organisms has been done shortly after the demonstration of the development of cutaneous hypersensitivity to Old Tuberculin or P.P.D. In contrast little appears to be known of the extent to which this increased resistance persists after the waning and ultimate disappearance of demonstrable cutaneous tuberculin sensitivity. This is important not only in assessing the relationship of sensitivity and resistance, but also in predicting what may be expected to follow human prophylactic vaccination. It is a matter of conjecture only whether increased resistance continues after the reversion to the Mantoux-negative state.

EXPERIMENTAL.

The guinea-pigs used in these experiments were all from the laboratory-bred stock at the Sir William Dunn School of Pathology, Oxford. The daily diet consisted of crushed oats, grass meal and bran, supplemented with cabbage. As all animals were left until they died from their tuberculous infection, closed bin cages with a wire grille above were used to minimise the risk of dissemination of infected material. These cages were changed, cleaned and sterilised at approximately weekly intervals.

Three experiments were carried out, each employing three groups of animals, as follows:

Group A: 12 guinea-pigs, unvaccinated.
Group B: 6 guinea-pigs, vaccinated 2 mg. B.C.G.
Group C: 6 guinea-pigs, vaccinated 2 mg. vole bacillus, strain OV191/3.

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Preparation of vaccines.

Both the B.C.G. and vole bacillus vaccines were suspensions made in sterile distilled water from ten-day cultures on Dorset's egg medium. They were used on the day of preparation. The B.C.G. strain was cultured on Dorset's egg medium from vaccine sent out from Copenhagen for human use. The strain of vole bacillus was that used for human vaccination and maintained in this laboratory.

Experiment 1.

The vaccinated animals in Groups B and C were tuberculin-tested with 0·2 ml. 1/10 Old Tuberculin at daily intervals after vaccination. Within seven days of becoming sensitive they were challenged with virulent bovine tubercle bacilli (0·5 mg. moist weight of culture subcutaneously), one control being challenged as each one vaccinated became Mantoux-positive.

Experiment 2.

In this experiment the animals which had been vaccinated with either B.C.G. or vole bacillus were not challenged with virulent bovine tubercle bacilli until they had lost their tuberculin sensitivity. Tuberculin testing, again with 0· 2ml. 1/10 Old Tuberculin, was carried out at approximately weekly intervals until a positive reaction was no longer elicited. As was to be expected, those which had received vole bacillus took far longer to revert to insensitivity to tuberculin. In this group also a control animal was challenged simultaneously with a vaccinated animal. After challenging, each animal was tested at intervals of two days with a 1/10 dilution of Old Tuberculin in order to establish in the animals vaccinated how rapidly tuberculin sensitivity reappeared.

Experiment 3.

In this experiment challenging was delayed until the animals had remained tuberculin-negative for not less than six months. As with the guinea-pigs in Experiment 2, tuberculin testing after challenging was carried out on alternate days on the infected animals until conversion was established.

As previously stated, all animals after challenging were left until they died from their infection. The animals were weighed at weekly intervals for three weeks after challenging and thereafter fortnightly.

Strain of challenging organism.

The culture used for challenge in all experiments was a bovine strain of *Myco. tuberculosis* named " Branch." This organism, which has been used in much of the experimental work done in this laboratory, was isolated from a fatal case of generalised tuberculosis in a human subject. The organism is a typical dysgonic bovine tubercle bacillus and has been repeatedly shown to be highly virulent for experimental animals. A dose of 0·5 mg., when given subcutaneously, kills guinea-pigs in 50–60 days, and in a dose of 0·01 mg. given intravenously kills rabbits in an average of 21 days. The disease in both animals is a typical generalised tuberculosis.

RESULTS.

The results presented in Table I are an extension of the investigations carried out by Wells and Brooke (1940). In the present series the animals were not

TABLE I.—*Experiment* 1.

Guinea-pig.	Time taken for cutaneous sensitivity to appear after vaccination with vole bacillus.		Survival time.	
	Days.		*Days.*	
1	8		147	
2	6		172	
3	10		198	
4	12		149	
5	10		155	
6	8	*Average* 9·0	161	*Average* 163·7
	Time taken for cutaneous sensitivity to appear after vaccination with B.C.G.			
	Days.			
1	10		113	
2	8		97	
3	10		103	
4	12		119	
5	10		115	
6	8	*Average* 9·7	95	*Average* 106·0
	Time taken for cutaneous sensitivity to appear after infection with strain "Branch".			
	Days			
1	6		47	
2	6		59	
3	8		72	
4	6		45	
5	8		64	
6	8		65	
7	6		42	
8	8		58	
9	6		48	
10	8		51	
11	6		41	
12	8	*Average* 7·0	49	*Average* 53·6

killed after eleven weeks but were left until they died. They nevertheless confirm the previous work in showing that guinea-pigs receiving prophylactic vaccination with vole bacillus are much better protected than those receiving the same dose of B.C.G., the vole-vaccinated group living on an average 58 days longer than those vaccinated with B.C.G. and 110 days longer than the control unvaccinated animals. It will be noticed that sensitivity developed 2–3 days later after inoculation with vole bacillus or B.C.G. than after infection with the virulent challenging strain.

Table II shows the results of challenging after skin sensitivity due to vaccination has disappeared. The animals in the group vaccinated with vole bacillus survived for approximately the same time when infected with virulent tubercle bacilli in both Expt. 1 and 2; resistance to infection was the same whether the challenging dose was given immediately after tuberculin sensitivity had

TABLE II.—*Experiment* 2.

Guinea-pig.	Time taken for cutaneous sensitivity to reappear after infection of vole-vaccinated group. (*Days*)	Survival time (*Days.*)
1	4	168
2	4	187
3	4	155
4	4	178
5	4	140
6	4	142
	Average 4·0	*Average* 161·7
	Time taken for cutaneous sensitivity to reappear after infection of B.C.G.-vaccinated group. (*Days.*)	
1	6	98
2	8	84
3	4	79
4	6	86
5	4	103
6	6	93
	Average 5·7	*Average* 90·5
	Time taken for cutaneous sensitivity to appear after infection of control group. (*Days.*)	
1	6	64
2	8	58
3	8	49
4	8	59
5	6	81
6	6	70
7	6	64
8	8	59
9	6	60
10	8	55
11	8	48
12	8	61
	Average 7·2	*Average* 60·7

developed or after sensitivity had disappeared. The mean survival time in those animals vaccinated with B.C.G. was 15·5 days less in the group which were not challenged until sensitivity had disappeared compared with those which were challenged as soon as sensitivity had developed. The statistical significance of this difference is doubtful, but it will be noted that the mean survival time of the B.C.G.-vaccinated animals in both experiments is substantially less than that of the vole-vaccinated animals.

Of considerable interest in the data in Table II is the comparatively rapid reappearance of tuberculin sensitivity after challenging in the group vaccinated with vole bacillus. In all animals in this group sensitivity had reappeared after four days, i.e., on the second testing undertaken. An average of nearly six days was required to re-establish sensitivity in the B.C.G.-vaccinated group.

As shown in Table III, six months after the disappearance of cutaneous sensitivity there can be little doubt from the average survival times that the resistance conferred by the initial vaccination with B.C.G. has almost disappeared. Indeed, some of the B.C.G. animals died before some control unvaccinated guinea-pigs. In marked contrast the survival time of those vaccinated

with vole bacillus remained almost unchanged at all three stages at which they were challenged.

TABLE III.—*Experiment* 3.

(six months after disappearance of sensitivity).

Guinea-pig.	Time taken for cutaneous sensitivity to reappear after infection of vole-vaccinated group.		Survival time	
	Days.		*Days.*	
1	4		159	
2	4		198	
3	4		144	
4	Died before challenging from intercurrent pneumonia			
5	4		162	
6	4	*Average* 4·0	138	*Average* 160·2
	Time taken for cutaneous sensitivity to reappear after infection of B.C.G.-vaccinated group.			
	Days.			
1	6		52	
2	8		86	
3	6		45	
4	4		75	
5	8		51	
6	6	*Average* 6·3	63	*Average* 62·0
	Time taken for cutaneous sensitivity to appear after infection of control group.			
	Days.			
1	Died before challenging; cause unknown			
2	6		73	
3	10		52	
4	8		63	
5	8		(26)*	
6	10		49	
7	6		60	
8	10		69	
9	Died before challenging from pneumonia			
10	8		62	
11	6		68	
12	8	*Average* 8·0	48	*Average* 60·5

* This animal died and at post-mortem had an intestinal obstruction. Early tuberculous lesions were found in the liver and spleen. It has been excluded from the average.

In animals which have been tuberculin-negative for six months, as in those challenged immediately after becoming so, sensitivity is re-established after four days in those vaccinated with vole bacillus, whereas in the B.C.G. group the time taken for sensitivity to re-develop after challenging increases as the time between loss of sensitivity and challenging lengthens.

It is pertinent to stress here that, owing to the markedly longer period taken for the vole-vaccinated groups in Expt. 2 and 3 to lose their cutaneous sensitivity than the B.C.G.-vaccinated group, as shown in Table IV, there is a fairly wide difference in age between the three groups, the vole group being the oldest and the B.C.G. group the youngest. The challenging of an unvaccinated control simultaneously with each vaccinated animal results in the age of the control

TABLE IV.—*Time taken for the Guinea-pigs Vaccinated in Experiments 2 and 3 to become Tuberculin-negative (0·2 ml. O.T. intracutaneously).*

Vole-vaccinated group.				B.C.G.-vaccinated group.			
Guinea-pig.		Days.		Guinea-pig.		Days.	
Expt. 2	1	287		*Expt.* 2	1	209	
	2	350			2	182	
	3	314			3	231	
	4	294			4	174	
	5	371			5	217	
	6	329			6	294	
Expt. 3	1	374		*Expt.* 3	1	217	
	2	287			2	245	
	3	301			3	203	
	4	392			4	228	
	5	334	*Average* 330·3		5	261	
					6	252	*Average* 226·1

group being intermediate. This difference is also reflected in the weight curves graphically presented later. However, Young (1951) has observed, and it is confirmed here, that mere age does not materially affect the susceptibility of the guinea-pig to experimental tuberculosis. The average age of the controls is widely different in the three experiments described here, but their survival time is almost the same in each experiment.

Post-mortem appearances.

Young (1951) suggested that in his experience animals vaccinated with the vole bacillus, when they do ultimately succumb to the challenging dose of virulent tubercle bacilli, exhibit a more extensive involvement of the viscera than those which have not been vaccinated. All our animals were subjected to very careful post-mortem examination, but it could not be confirmed that the animals vaccinated, either with vole bacillus or with B.C.G., exhibited more extensive lesions at death. It is true that from time to time an animal did show what appeared to be abnormally extensive infiltration of one or other organ; one, a B.C.G.-vaccinated animal in Expt. 2, was found at *post mortem* to have the spleen almost entirely replaced by caseous material. In general, however, the degree of tuberculosis was not more extensive in any of the three groups, nor did it differ from experiment to experiment.

Weight changes after vaccination and challenging.

Animals used in these experiments were weighed at regular intervals during the investigation. The results of these estimations are presented graphically below. In Expt. 1 weekly weighings of all guinea-pigs were made three weeks before vaccination. Thereafter for three weeks the weight was measured weekly and then fortnightly. Fig. 1 shows that following vole vaccination there is a drop in weight which takes six weeks to be regained. A similar though less severe and less protracted fall is observed after B.C.G. vaccination but, as is to be expected, control unvaccinated guinea-pigs receiving the same diet continue steadily to gain in weight. Although the vole bacillus in the dose used does not cause progressive tuberculosis, there is no doubt that on subcutaneous

injection it gives rise to a more severe local lesion than B.C.G. This can reasonably be supposed to have a systemic effect reflected here in the fall in weight.

Figs. 2 and 3 represent the average changes in weight in Expt. 2 and 3, after challenging when sensitivity has been lost in Expt. 2, and six months after its disappearance in Expt. 3. It will be clearly seen that in the groups vaccinated with vole bacillus a steady gain in weight up to sixteen weeks follows challenging. The rise appears somewhat steeper in those challenged immediately after the disappearance of sensitivity, the final fall being steeper in this group also. In those vaccinated with B.C.G., if challenged as in Expt. 2 when a skin reaction

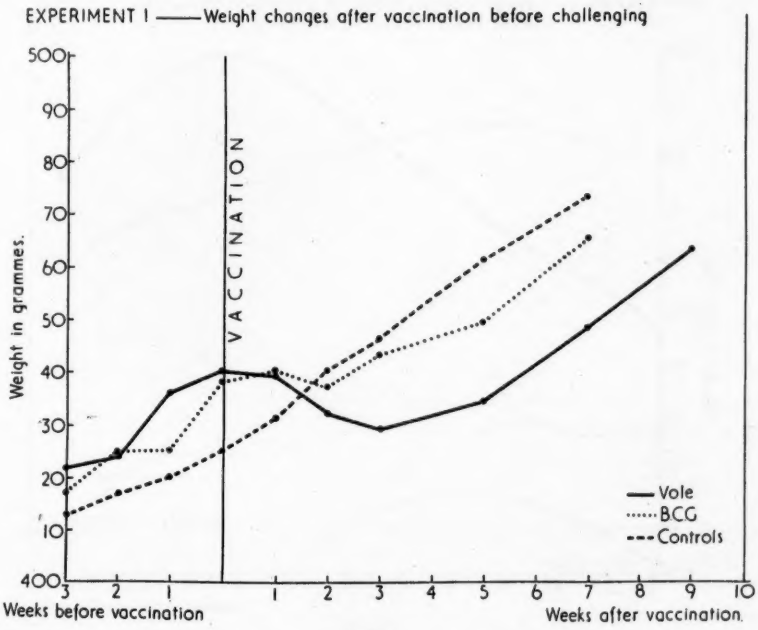

FIG. 1.

can no longer be elicited, there is a steady rise for seven weeks followed by an approximately equally steady decline, whereas in Expt. 3, when the challenge was delayed until six months after sensitivity had disappeared, the B.C.G. group show a weight curve approximating to that shown by the control unvaccinated animals after the challenge, confirming the almost complete loss of resistance in this group.

The rather wide difference in the average weight in the three groups in Expt. 2 and 3 is explained by the fact, already mentioned, that the animals receiving vole bacillus prophylaxis take far longer to lose their cutaneous sensitivity than those receiving B.C.G.; but, as a control unvaccinated animal was challenged contemporaneously with each of the vaccinated animals, the average weight of this group lies approximately mid-way between the two vaccinated groups.

DISCUSSION.

The relationship between tuberculin sensitivity and immunity has long been a subject of contention. The literature is well summarised by Wilson and Miles (1946). They give the evidence from animal experiment and epidemiological study which shows the complexity of the relationship and the difficulty of interpretation. The results of our experiments show that it is possible for immunity to exist without sensitivity. This statement may need modification if organ sensitivity can exist for a long period without skin sensitivity, but of this we have no evidence one way or another. It has, of course, long been known that

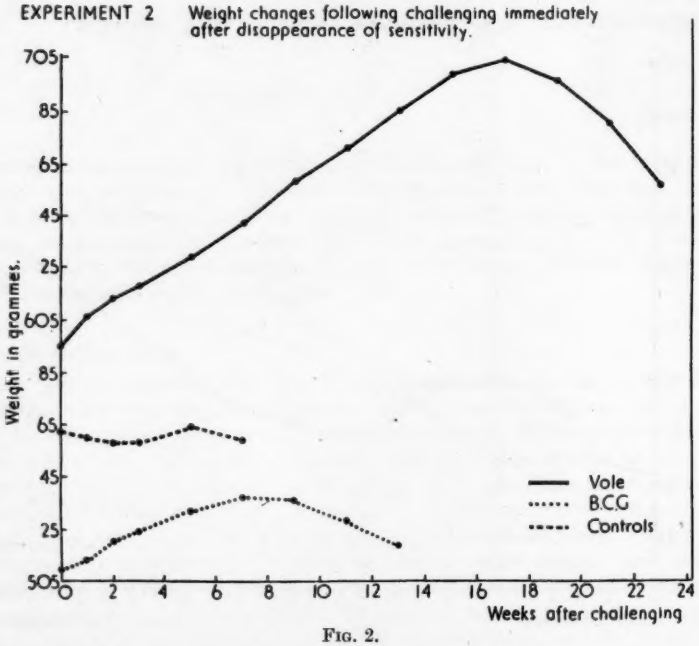

FIG. 2.

skin sensitivity in tuberculous guinea-pigs can be temporarily abolished or reduced by repeated injections of tuberculin, and the experimental evidence for separating sensitivity and immunity has largely been based on such de-sensitisation. Those who hold the view that sensitivity and immunity to subsequent virulent infection are different expressions of the immunity phenomenon claim that de-sensitisation by tuberculin is incomplete, and that sensitivity of the organs remains even though skin sensitivity may be temporarily suppressed. The fact that skin sensitivity was allowed to disappear spontaneously in our animals does not, of course, prove that organ sensitivity disappeared at the same time. It seems likely, however, that in Expt. 3, after a period of six months during which skin sensitivity was completely absent, organ sensitivity had also disappeared. Yet those animals in Expt. 3 which had been inoculated with the vole bacillus retained unimpaired their induced resistance to virulent infection

six months after skin sensitivity had disappeared. The animals inoculated with B.C.G. had lost their immunity to infection six months after sensitivity was lost, but we think this can be explained by the greater antigenic potency of the vole bacillus compared with B.C.G.

There is no evidence of any quantitative relationship in our experiments between sensitivity and immunity. In Expt. 1, where the vaccinated animals were challenged as soon as sensitivity had developed, the unvaccinated controls developed sensitivity earlier than those vaccinated with B.C.G., which survived

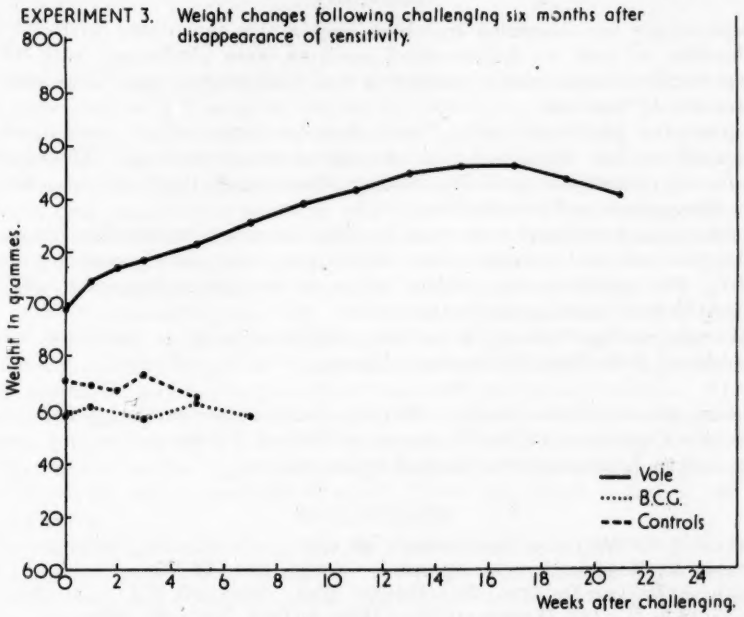

FIG. 3.

longer. In Expt. 2 and 3, where the challenge was delayed until skin sensitivity had disappeared, sensitivity reappeared first in those animals which had been vaccinated with vole bacillus and these animals survived longest. Moreover in these last two experiments sensitivity appeared more quickly in the vaccinated animals than in the unvaccinated controls.

The duration of sensitivity is considerably greater after vaccination with the vole bacillus than B.C.G., as shown in Table IV. The vole bacillus has a higher " virulence " for the guinea-pig than B.C.G. and, if given in sufficiently large doses will produce progressive disease. This greater " virulence " is also shown if the vole bacillus is used in human vaccination. The number of bacilli needed to give comparable tuberculin sensitivity is very much smaller than if B.C.G. is used. It seems probable that the vole bacillus survives longer in the animal body than does B.C.G. and that sensitivity remains high only as long as living bacilli are present.

It seems to us that there may be some application of these experiments to vaccination in man. It is customary in B.C.G. vaccination to re-vaccinate as soon as skin sensitivity is lost, and there seems to be justification for this from the work presented here. On the other hand, the fact that immunity after vaccination with the vole bacillus is as high six months after skin sensitivity is lost as at any intervening period after sensitivity has first developed suggests that re-vaccination in man may not be so necessary.

SUMMARY.

Experiments are described in which guinea-pigs inoculated with B.C.G. or vole bacillus as well as uninoculated controls were challenged with virulent tubercle bacilli as soon as skin sensitivity had disappeared, and six months after skin sensitivity was lost.

Guinea-pigs inoculated with B.C.G. survive longer when challenged after skin sensitivity has developed than do uninoculated controls. This increased resistance to infection is probably waning when sensitivity is lost and has completely disappeared six months later.

Guinea-pigs inoculated with vole bacillus survive considerably longer than the B.C.G.-inoculated animals when challenged after skin sensitivity has developed. The resistance to virulent infection remains unimpaired both when sensitivity is lost and six months later.

The relationship between sensitivity and immunity is discussed and the independence of the two phenomena is shown.

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THE ABSORPTION OF OLIVE OIL FROM THE INTESTINE IN RATS DEPRIVED OF PTEROYLGLUTAMIC ACID, BIOTIN AND PYRIDOXIN.*

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It has been suggested that sprue is a deficiency disease and that the steatorrhoea associated with it may be caused by lack of B_2 vitamins in the intestine (Stannus, 1942; Leishman, 1945; Angulo and Spies, 1947). Investigations have therefore been undertaken to determine the effect of various B_2 vitamins on the absorption of fat. Frazer (1949*a*) has shown that riboflavine, nicotinamide, pyridoxin and pantothenic acid are probably without effect on fat absorption. Attention was therefore directed to pteroylglutamic acid and biotin, two members of the B_2 group not covered by the previous study. It has been shown that deficiency of pteroylglutamic acid and/or biotin does not impair the ability of rats to absorb oleic acid (Woodruff, 1950*a*). Accordingly, the effect of deficiency of these substances on the absorption of neutral fat in rats has been investigated. Olive oil was selected for use in this connection as it is a mixture of neutral fats, is readily available, easy to handle and is a naturally occurring product. The work of Frazer (1949*b*) gave added interest to the experiments. He suggested that the underlying fat absorption fault in sprue is a defect in the absorption of neutral fat, that the unabsorbed neutral fat is hydrolysed in the intestine producing long chain fatty acids, to the presence of which Frazer attributes many of the symptoms of sprue.

The effect of pyridoxin deficiency on the rat's ability to absorb olive oil was further studied as it has been suggested that pyridoxin might be concerned in the mobilisation and transport of fat (Birch, 1938). Stannus (1942) considered that deficiency of pyridoxin might be a factor in the aetiology of sprue.

METHODS.

The experimental procedure, the type of animals used, their rations, care, and the method of inducing in them deficiencies of pteroylglutamic acid and biotin have been described (Woodruff, 1950*a*). Halliday and Evans (1937) showed that pyridoxin deficiency in rats can be produced by feeding them on a diet of vitamin-free casein, cotton seed oil, sucrose and vitamin supplements other than pyridoxin. In the present investigation vitamin-free arachis oil (British Drug Houses) was substituted for the cotton seed oil and vitamin-free maize starch for the sucrose. Succinylsulphathiazole (2 per cent) was incorporated in the diet as it has been shown that it depresses the synthesis of B vitamins in the intestine of rats (Wright and Welch, 1944). The vitamin supplements provided to the control animals were those described by Woodruff (1950*a*). To the pyridoxin-deficient group these supplements less pyridoxin were given.

The deficiency being fully established, the animals after 24 hours' starvation, were anaesthetised with 1–2 ml. of a 2 per cent urethane solution in normal saline and were then given 1 ml. olive oil by stomach tube. The distribution of the oil was studied $6\frac{1}{2}$ hours later by the methods previously described.

* This paper formed part of a thesis for which the Ph.D.London was awarded.

RESULTS.

In the Table are shown the rates of absorption of olive oil in healthy control rats and in those deprived of pyridoxin, pteroylglutamic acid, biotin, and both pteroylglutamic acid and biotin.

TABLE.—*Distribution of Olive Oil in Rats Receiving Deficient Diets.*

	Amount of olive oil leaving stomach during experiment (mg. per hour).		Absorption of olive oil reaching small intestine (percentage absorbed per hr.)		Fat recovered from caecum and colon (mg.).		Number of experiments.	Mean weight of animals. (g.).
	Mean.	Standard deviation.	Mean.	Standard deviation.	Mean.	Standard deviation.		
Control group (animals receiving full vitamin supplements)	37·45	±13·93	8·20	±1·69	50·85	±20·56	7	93
Pteroylglutamic-deficient animals	59·39	±19·41	9·09	±2·13	52·33	±10·92	6	82
Biotin-deficient animals	63·13	±21·78	7·38	±1·93	48·71	±13·19	7	94
Pteroylglutamic acid- and biotin-deficient animals	48·05	±15·06	7·41	±1·80	57·33	±30·46	9	84
Pyridoxin-deficient animals	62·07	±14·66	6·42	±2·24	71·28	±42·49	7	92

A comparison of all the possible pairs of means in the series and application of the " t " test of significance shows that there is no significant difference in the rates of absorption of olive oil in the various groups of animals. The greatest difference is between the means for the pteroylglutamic acid-deficient and pyridoxin-deficient groups. This difference is $9{\cdot}09-6{\cdot}42 = 2{\cdot}67$; it gives a value for " t " of 2·02 and $0{\cdot}05 < P < 0{\cdot}1$.

The rates at which olive oil left the stomach of animals in the pyridoxin- and biotin-deficient groups are significantly greater than those in the control group. The difference between the mean rates in the control group and in the biotin-deficient group is $63{\cdot}13-37{\cdot}45 = 25{\cdot}68$, giving a value for " t " of 2·43, and $0{\cdot}05 > P > 0{\cdot}02$. Between similar rates for the control group and the pyridoxin-deficient group, the difference is $62{\cdot}08-37{\cdot}45 = 24{\cdot}63$, giving a value for " t " of 2·98 and $0{\cdot}01 < P < 0{\cdot}02$. Whatever the cause of this increased gastric motility, it was not such as to lead to a rate of absorption of olive oil significantly different from that in the other groups, although the rates of absorption in these two groups were lower than in the other three.

Insignificant differences are found on comparison of all the possible pairs of figures relating to amounts of fat recovered from the caecum and colon of animals at the end of the experiments. This provides confirmatory evidence that there was no marked impairment in absorption of fat from the small intestine of the animals, as otherwise one would expect an abnormally large amount of fat to pass through the small intestine and reach the caecum. An exception to this statement might be caused by depression in intestinal motility should it occur under the conditions of the experiments; the evidence suggests, however, that motility, if affected at all, is increased.

DISCUSSION.

These experiments indicate that deficiency of pteroylglutamic acid, biotin or pyridoxin does not influence the ability of rats to absorb olive oil from the intestine. Deficiency of pteryolglutamic acid and/or biotin also appears to be without effect on the absorption of oleic acid from the intestine of rats (Woodruff, 1950*a*). Further experiments in this series (Woodruff, unpublished) show that in pyridoxin deficiency in the rat the rate of absorption of oleic acid is normal. Frazer (1949*a*) reported experiments in which the histology of the intestines was studied in healthy rats given mixtures of olive oil and thiamine, riboflavine, nicotinamide, pyridoxin, pantothenic acid, and choline. From observations on the distribution, size and number of fat droplets in the intestinal cells, Frazer concluded that thiamine, riboflavine, nicotinamide, pyridoxin and pantothenic acid were without effect on the absorption of olive oil in these animals. Black, Bound and Fourman (1947) found that administration of nicotinic acid or riboflavine to sprue patients failed to improve fat absorption, and Woodruff (1950*b*) found that pteroylglutamic acid similarly failed in such patients. Fat balance studies in sprue patients and experiments in rats in which pure vitamins have been used have, therefore, so far failed to reveal any evidence that vitamins are directly concerned in the process of fat absorption. In view of these findings it seems to be unlikely that sprue is primarily a nutritional disease.

SUMMARY.

Rats were maintained on a standard diet, and to a series of controls and to others deprived of pteroylglutamic acid, biotin and pyridoxin, 1 ml. olive oil was administered. After 6½ hours the distribution of fat in the intestine was determined.

The amounts of olive oil absorbed after reaching the small intestine varied in the different groups from 6·42–9·09 per cent per hour, these differences being statistically insignificant.

The amounts of fat recovered from the caecum at the end of the experiments were not significantly increased in any group.

The rate of absorption of olive oil was thus apparently unaffected by these deficiencies.

Evidence at present available suggests that the defect in fat absorption in sprue is not caused by deficiency of B vitamins.

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